QIAGEN

The QIA*expressionist*

QIA*express*:

The high level expression & protein purification system

for

- **functional studies**
- **epitope screening**
- **antibody induction**
- **enzyme expression**
- **diagnostic purposes**
- **small peptide production**
- **secreted proteins**

Summer 1992

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Written and compiled by Joanne Crowe, QIAGEN Inc.
and Karsten Henco, DIAGEN GmbH.

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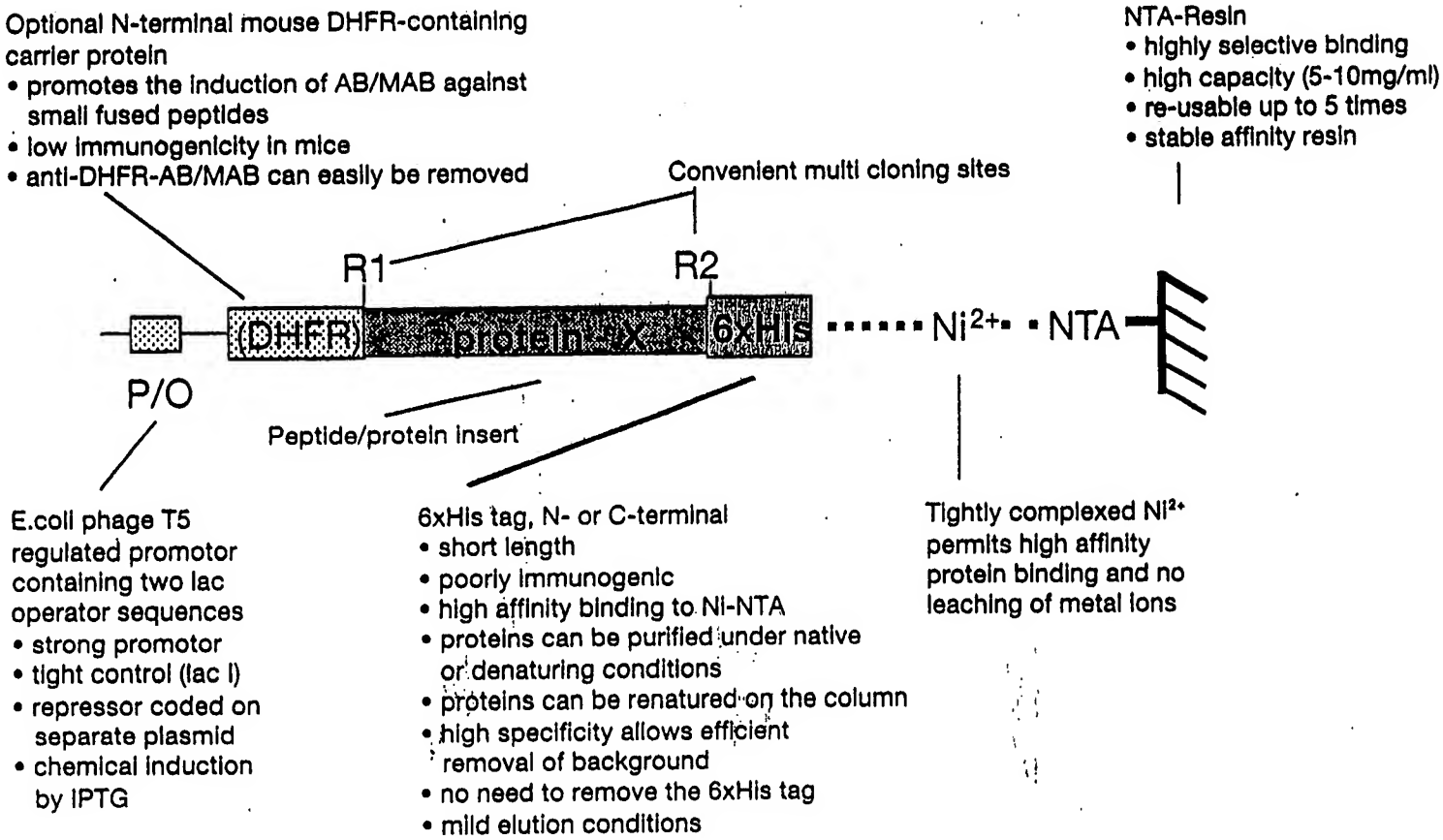


Figure 1: Features of the QIAexpress system.

Introduction: The QIA*express* System

The QIA*express* system is a fast and versatile tool for the expression and purification of recombinant proteins and antigenic peptides. QIAexpress is based on the remarkable selectivity of our patented Ni-NTA resin for proteins with an affinity tag consisting of just six consecutive histidine residues (6xHis tag).

The affinity of Ni-NTA resin for the 6xHis tag is so strong that it allows contaminating proteins to be washed away under extremely stringent conditions, which are nonetheless very mild towards the tagged protein, and do not affect the specific binding. This permits purification of proteins from less than 1% to greater than 95% homogeneity in just one step (Janknecht *et al.*, 1991). Only Ni-NTA resin, available exclusively from DIAGEN GmbH (Germany), QIAGEN Inc. (USA), or their distributors, can bring this kind of power to protein purification.

QIA*express* combines the advantages of a high-level bacterial expression system with the resolving power of the 6xHis/Ni-NTA purification system to provide:

- One-step purification of recombinant proteins under either native or denaturing conditions.
- Efficient purification of proteins from very dilute solutions.
- Easily reversible binding that allows elution of the protein under very mild conditions.
- Pure protein products ready for direct use in a variety of procedures.
- High-level bacterial expression of recombinant proteins with either N- or C-terminal 6xHis affinity tags.
- No time consuming protease digestions to remove the affinity tag.
- Easy adaptation of the purification system to other expression systems.
- An inexpensive and stable affinity resin, which may be reused up to five times.

QIA*express* Kits contain everything necessary for the expression and purification of recombinant proteins, and are available in five types. The choice of kit depends on the type of recombinant protein to be constructed. QIA*express* pQE vectors, *E. coli* host strains and Ni-NTA resin are all also available separately. Please see Appendix H for a complete list of pQE vectors and QIA*express* products.

Section A - General Information

I. The Expression System

1. QIA*express* pQE Vectors

The pQE expression vectors provide high-level expression in *E. coli* of proteins or peptides containing a 6xHis affinity tag. The tag may be placed at the N-terminus of the protein to create a Type IV construct; at the C-terminus of the protein to create a Type III construct; or at the C-terminus of a protein utilizing its original ATG start codon, to create a Type ATG construct (Fig. 2). If small peptides are being synthesized, they can be fused to mouse DHFR to create a Type II construct, where the poorly immunogenic DHFR stabilizes the peptide during expression, and enhances its antigenicity.

The pQE plasmids belong to the pDS family of plasmids (Bujard *et al.*, 1987) and were derived from plasmids pDS56/RBSII and pDS781/RBSII-DHFRS (Stüber *et al.*, 1990). They contain the following elements:

- An optimized, regulable promoter/operator element, consisting of the *E. coli* phage T5 promoter and two *lac* operator sequences.
- A synthetic ribosome binding site, RBSII, designed for optimal mRNA recognition and binding.
- Optimized 6xHis affinity tag coding sequence.
- The mouse DHFR coding sequence (Chang *et al.*, 1978; Masters and Attardi, 1983) (in Type II vectors only).
- A multi-cloning site.
- Translation stop codons in all reading frames.
- The transcriptional terminator 't_o' from phage lambda (Schwarz *et al.*, 1987).
- The replication region and the gene for ß-lactamase of plasmid pBR322 (Sutcliffe, 1979).

2. Regulation of Expression - pREP4 Repressor Plasmid

Expression from the promoter/operator region is extremely efficient, and can only be prevented by the presence of high levels of *lac* repressor. The *E. coli* host cells in the QIA*express* system contain multiple copies of the plasmid pREP4, which carries the *lacI* gene (Farabaugh, 1978) encoding the *lac* repressor. The multiple copies of pREP4 present in the host cells ensure high levels of *lac* repressor and tight regulation of protein expression. Due to mutations in its ribosome binding site, pREP4 produces less NEO than similar plasmids, so it requires only 25 µg/ml of kanamycin for selection. The plasmid is compatible with all plasmids carrying the ColE1 origin of replication.

Expression from pQE vectors is rapidly induced by the addition of IPTG, which inactivates the repressor and clears the promoter. The special 'double operator' system (Fig. 1) in the pQE expression vectors, in combination with the high levels of *lac* repressor provided by pREP4, permits some control over the level of expression. The high levels of *lac* repressor can be reduced with low amounts of IPTG, leading to only partial clearing of the promoter and concomitant low level transcription if desired.

3. *E. coli* Host Strains

Any *E. coli* host strain containing both the expression (pQE) and the repressor (pREP4) plasmid can be used for the production of recombinant proteins. QIA*express* uses the K-12 derived *E. coli* strains M15[pREP4] (Villarejo and Zabin, 1974) and SG13009[pREP4] (Gottesman *et al.*, 1981), which allow high level expression and are easy to handle. SG13009[pREP4] gives levels of expression that are toxic to the cells for most proteins, but may be useful for the production of proteins that are expressed poorly in M15[pREP4]. Both strains are: Nal^S Str^S rif^S, lac^- ara^- gal^- mtl^- F^- $recA^+$ uvr^+.

E. coli strains that contain the $lacI^q$ gene, such as JM109 and TG1, are well suited for storing and propagating the pQE plasmids. This gene has a mutated promoter, and produces up to 10-fold more *lac* repressor than the wild-type gene. Strains carrying this gene produce enough *lac* repressor to block expression, without carrying the pREP4 plasmid. They can also be used as expression hosts, although they will provide less tightly regulated expression than strains carrying the pREP4 plasmid. This is critical in cases where the expressed protein is toxic to the cell, and 'leaky' expression before induction may result in poor culture growth or in a selection of a mutated pQE vector.

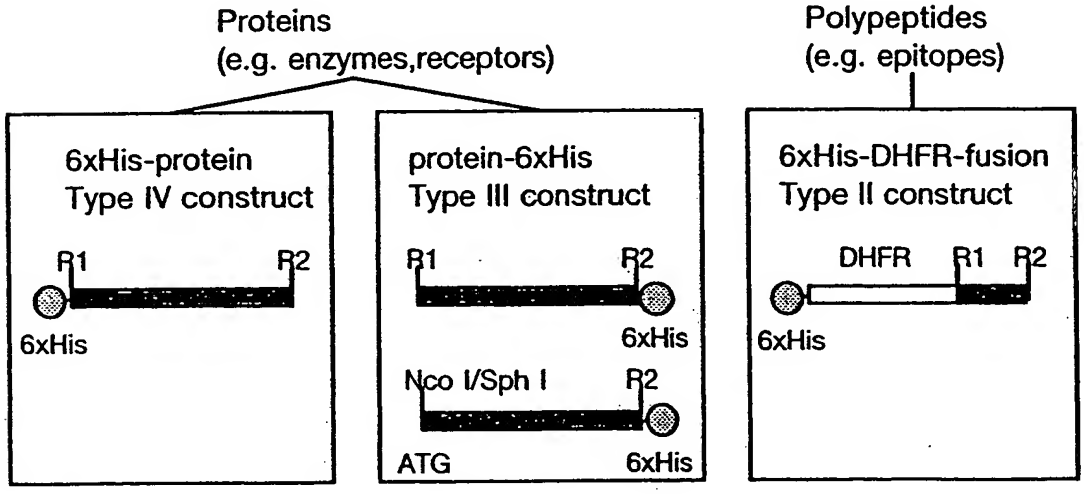


Figure 2: QIAexpress constructs.

II. The Purification System

The QIA*express* purification system employs a novel metal chelate adsorbent — Ni-NTA resin — which provides an elegant yet simple one-step method for rapid protein purification (Hochuli *et al.*, 1987). The power of the QIA*express* system is based on the remarkable affinity of Ni-NTA resin for proteins and peptides that contain six consecutive histidine residues — the 6xHis affintiy tag — at either their N- or C-terminus (Hochuli *et al.*, 1988).

1. The Ni-NTA Resin

Immobilized metal chelate affinity chromatography, first used to purify proteins in 1975 (Porath *et al.*, 1975), has become a widely used technique. Until the development of NTA (nitrilo-tri-acetic acid), the chelating ligand iminodiacetic acid (IDA) was charged with metal ions such as Zn^{2+} and Ni^{2+}, and then used to purify a variety of different proteins and peptides (Sulkowski, 1985). However, the IDA ligand has only three chelating sites, and does not bind metal ions containing six coordination sites tightly. The weak binding allows the metal ions to be washed out of the resin upon loading with strong chelating proteins and peptides, or during the washing of the bound proteins. This results in low yields, impure products, and heavy metal contamination of purified proteins.

NTA resin is a new chelating adsorbant developed at Hoffmann-La Roche (Basel) in order to overcome these problems. The NTA ligand has four chelating sites which can interact with metal ions. Thus NTA occupies four of the six ligand binding sites in the coordination sphere of the Ni^{2+} ion, leaving two sites free to interact with the 6xHis tag (Fig. 3). This allows NTA to bind the metal ions far more stably than other available chelating resins, retaining the ions under a wide variety of conditions (Hochuli, 1989). As a result, this unique, patented resin binds 6xHis-tagged proteins 1000x more tightly than IDA (Hochuli *et al.*, 1989), allowing for more discriminating separation of contaminating proteins. This extremely high affinity interaction between the 6xHis tag and Ni-NTA resin permits the purification of proteins from less than 1% to greater than 95% homogeneity (Janknecht *et al.*, 1991).

Ni-NTA resin is available exclusively from QIAGEN Inc. (USA), DIAGEN GmbH (Germany) and their distributors.

2. Protein Binding

Proteins containing one or more 6xHis affinity tags, located at either the amino or carboxyl terminus of the protein, bind to the Ni-NTA resin with an affinity ($Kd = 10^{-13}$, pH 8.0) far greater than the affinity between most antibodies and antigens, or enzymes and substrates. This means that any host proteins that bind non-specifically

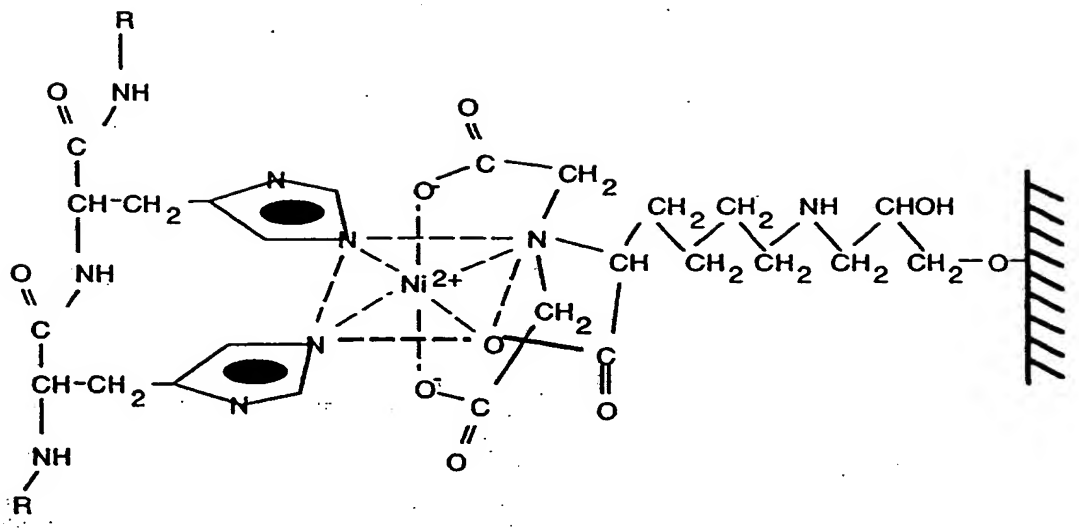


Figure 3: Interaction between 6xHis tag and Ni-NTA resin.

to the NTA resin can be easily washed away under relatively stringent conditions, without affecting the binding of the 6xHis-tagged proteins. Zinc finger proteins for example, will bind to the Ni-NTA resin, but can be easily discriminated from the proteins carrying the 6xHis tag, which bind more tightly. The high binding constant also allows proteins in very dilute solutions, such as those expressed at low levels or secreted into the media, to be efficiently bound to the resin.

The binding of tagged proteins to the resin does not require any functional protein structure and is thus unaffected by strong denaturants such as 6 M guanidine hydrochloride or 8 M urea. This means that, unlike purification systems which rely on antigen/antibody or enzyme/substrate reactions, Ni-NTA can be used to purify almost any protein — even those that are insoluble under non-denaturing conditions.

The stability of the 6xHis/Ni-NTA interaction in the presence of low levels of ß-mercaptoethanol (1 - 10 mM) prevents the co-purification of host proteins that may have formed disulfide bonds with the protein of interest during cell lysis.

The presence of detergents such as Triton®X-100 and Tween®-20 (0.1 - 1%), or high salt concentrations (0.1 - 1 M), also has no effect on protein binding. This facilitates the complete removal of proteins that would normally co-purify due to various non-specific interactions. Nucleic acids that might associate with certain DNA and RNA binding proteins can also be efficiently removed without affecting the recovery of the tagged protein.

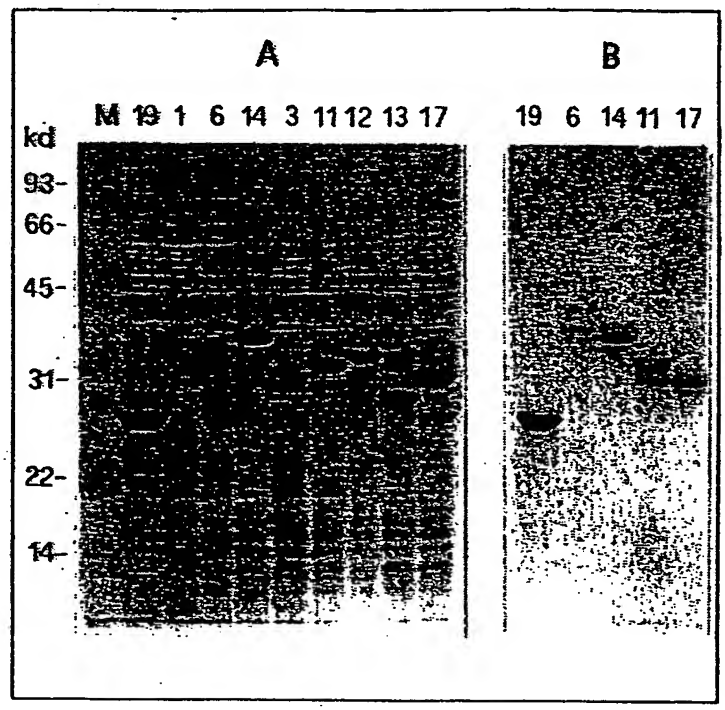


Figure 4: Expression and purification of malaria circumsporozoite antigens with QIAexpress.
A. SDS-Page analysis of recombinant 6xHis-tagged CS proteins, 5 hours after induction with IPTG.
B. SDS-Page analysis of recombinant CS proteins after purification on Ni-NTA resin according to the standard protocol, and elution at pH 5.9. Each protein is more than 80% pure.
D. Stüber, Hoffmann - La Roche, Basel, Switzerland.

3. Protein Elution

Elution of the tagged proteins from the column can be achieved by several methods. Reducing the pH will cause the histidine residues to become protonated, and to dissociate from the Ni-NTA. Monomers are generally eluted at approximately pH 5.9, while aggregates and proteins that contain more than one 6xHis tag elute around pH 4.5.

Elution can also be achieved by competition with imidazole (Fig. 5), which binds to the Ni-NTA and displaces the tagged protein. Low levels of imidazole can also be used to selectively elute contaminants that bind less strongly to the resin (Janknecht *et al.*, 1991).

4. Binding Capacity

The Ni-NTA resin is composed of a high surface concentration of NTA ligand attached to Sepharose® CL-6B, sufficient for the binding of approximately 5 - 10 mg of 6xHis tagged protein per ml of resin. The Ni-NTA resin can be reused 3 - 5 times for purification of the same protein, and is very stable and easy to handle. It retains full activity even after prolonged storage.

5. The 6xHis Affinity Tag

The affinity tag that binds to the Ni-NTA resin consists of just six consecutive histidine residues. Its small size means that there is minimal addition of extra amino acids to the recombinant protein. It is non-immunogenic — or at most, very poorly immunogenic — in all species except some monkeys. It is uncharged at physiological pH and generally does not affect the secretion, compartmentalization, or folding of the protein to which it is attached. In over 150 proteins purified using this system at Hoffmann-La Roche, they have never found the 6xHis tag to interfere with the structure or function of the purified protein. This has been examined for a wide variety of proteins, including enzymes (Döbeli *et al.*, 1990), transcription factors (Janknecht *et al.*, 1991) antigens (Stüber *et al.*, 1990, Takacs and Gerard, 1991), and membrane proteins (D. Meyer, personal communication), to name just a few.

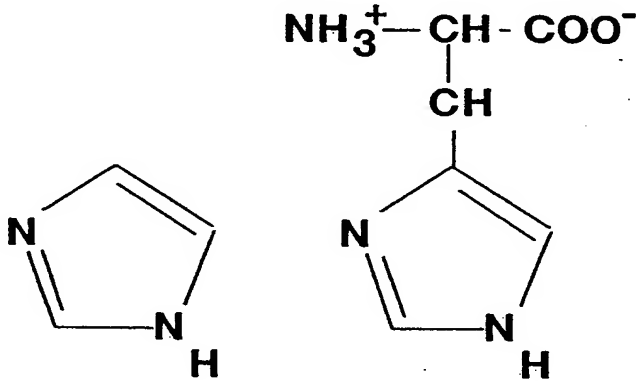


Figure 5: Structures of imidazole and histidine.

For all of the above reasons there is rarely any need to remove the 6xHis affinity tag from the recombinant protein after purification. Proteolytic removal of affinity tags is time-consuming, often inefficient, and rarely necessary with the QIA*express* system. If it is desirable for some reason to remove the tag (from very small proteins, for example, where the tag represents a large portion of sequence) a protease cleavage site can be inserted easily into the construct (see Section III.9).

The small size of the 6xHis tag makes it ideally suited for inclusion in a variety of other expression systems, whether prokaryotic, mammalian, yeast, baculovirus or any other hosts are used. A short sequence coding for the 6xHis tag, $(CAT\ CAC)_3$, can be introduced into the expression construct via PCR, *in vitro* mutagenesis, or fragment insertion (see Section III. 9).

III. Choosing a QIA*express* Construct

There are several ways to create a QIA*express*-type expression construct (Fig. 2). The 6xHis tag may be placed at the N-terminus of the recombinant protein (Type IV); at the C-terminus (Type III); at the N-terminus, in conjunction with the sequence for mouse DHFR (Type II); or at the C-terminus, with the protein beginning with its natural ATG start codon (Type ATG) in order to maintain an authentic leader sequence.

Type IV constructs, which place the 6xHis tag at the N-terminus of the protein, are the most commonly used, and are generally the easiest to prepare. They do not require that the entire coding sequence be accurately determined, since the pQE vectors have stop codons in all three reading frames. C-terminal tags will not be expressed if the coding sequence contains an undetected frame shift, or translational stop codon. Moreover, in the pQE expression vectors, Type IV proteins are often expressed 2-4 times more efficiently than proteins with a C-terminal affinity tag (S. Le Grice, personal communication).

While an N-terminal 6xHis affinity tag is often the best choice, it is wise to consider whether a different construct might be more appropriate, before making a decision. The following details should be considered:

1. Use of Recombinant Proteins

The choice of construct will depend to some extent on the intended use of the recombinant protein. For many applications it is desirable to minimize the amount of extra amino acids added to the protein in addition to the 6xHis tag. This can be achieved by creating a Type ATG construct, or by careful cloning into one of the Type IV vectors. If the number of additional amino acids introduced by the cloning procedure is not so important, then it is sufficient to clone into the most convenient restriction sites of the multi-cloning site.

2. Denaturing or Non-Denaturing Purification

If the protein is to be purified under native conditions, it is best to place the 6xHis tag at the end of the protein that is most likely to be exposed. If nothing is known about the folding, it may be preferable to place the 6xHis tag at the N-terminus, since the C-termini of proteins are more likely to be inaccessible.

3. Size of Recombinant Proteins

Very small proteins and peptides are sometimes difficult to express stably in *E. coli*, because they cannot fold correctly and are often subject to proteolytic degradation. These small peptides can be stabilized by synthesizing them fused to a large protein like

mouse DHFR, in a Type II construct (Fig. 2). DHFR is poorly immunogenic in mice and rats, and enhances the general antigenicity of the peptides to which it is attached by allowing them to fold. It also protects them from proteolytic attack after immunization.

Very long recombinant proteins may be subject to premature termination. Placing the 6xHis tag at the C-terminus permits selection of full length proteins.

4. Codon Usage

Some proteins are poorly expressed in *E. coli* if they contain rarely used codons, which often cause premature termination of translation (Grosjean & Fiers, 1982). This can result in a variety of truncated protein products, particularly from large recombinant proteins. Placing the 6xHis affinity tag at the C-terminus will allow full length proteins to be purified away from the shorter contaminants.

5. Internal Start Sites

Placement of the 6xHis tag at the N-terminus of the protein prevents the co-purification of short proteins which can arise due to initiation of translation 3′ to the correct start site, within the coding region. Such internal starts arise when there is a ribosome binding consensus sequence (Shine-Dalgarno sequence) 5′ to an internal ATG or GUG codon. Internal starts can be avoided by mutation of any potential Shine-Dalgarno sequence in the coding region followed by a start codon without affecting the protein sequence.

6. Expression of Difficult Proteins

Some genes contain regions which interfere at the RNA level with the interaction between the *E. coli* ribosome and the ribosome binding site provided by the expression plasmid. This can create a severe inhibition of protein synthesis. In most cases this negative effect can be overcome by modifying the 5′ end of the gene to make it more A/T rich, or by expressing it downstream of a fusion partner such as DHFR (a Type II construct). Placing the 6xHis tag sequence at the 5′ end of the gene often increases expression levels.

Ribosome binding sites should be removed from recombinant sequences before they are cloned into the pQE vectors, to avoid possible interference with expression.

7. Secretion of Proteins

Secretion of proteins is often used to keep them soluble, however, yields are generally lower than for proteins which remain cytoplasmic. In addition, secretion often causes instability and problems with folding for proteins which are normally cytoplasmic.

QIAexpress can be used to purify most proteins, even from inclusion bodies, so it is generally not necessary to secrete them. If secretion is desirable — to encourage proper folding in some proteins, for example, or to keep toxic proteins extracellular — an appropriate signal sequence must be added to the N-terminus of the construct. In general it is preferable to place the 6xHis tag at the C-terminus, to prevent the possibility of its being lost during N-terminal processing. The 6xHis tag has no adverse effect on secretion.

8. Removal of the 6xHis Affinity Tag

In most cases there is no need to remove the 6xHis affinity tag from the purified proteins. If it must be removed, a protease cleavage site should be inserted between the 6xHis sequence and the N-terminus of the protein. Enterokinase recognizes the sequence DDDDK ($(Asp)_4$-Lys), and cleaves after the lysine. This sequence can be inserted into the construct by site-directed mutagenesis or PCR[1], after checking that it is not already present in the protein sequence (Fig. 6). Other proteine cleavage sites can be used alternatively.

Carboxypeptidase A can be used for the removal of C-terminal 6xHis tags. The enzyme efficiently removes aromatic C-terminal residues (Hochuli *et al.*, 1988) until it encounters a basic residue, at which point removal is terminated. Care should be taken to ensure that there are one or more basic residues (such as arginine) at the C-terminus of the recombinant protein.

9. Incorporation of the 6xHis Affinity Tag into Other Expression Vectors

The 6xHis tag can be easily incorporated into other expression vectors using various methods of mutagenesis, PCR, or fragment integration. The fragment below can be created from synthetic oligonucleotides with overlapping ends corresponding to the appropriate restriction site(s).

```
                His  His  His  His  His  His
5' XXXXX CAT CAC CAT CAC CAT CAC X 3'
      X GTA GTG GTA GTG GTA GTG XXXXX
```

[1]The Polymerase Chain Reaction (PCR) process is covered by U.S. Patents owned by Hoffmann-La Roche Inc. or Perkin Elmer Cetus.

10. Use of Transcription Vectors

There are many transcription vectors which seem at first glance to be useful as expression vectors. They should not be used for expression, however, because they and their transcripts are present in the cells at such high copy number that they accentuate the problems associated with toxic proteins and tight control of the promoters.

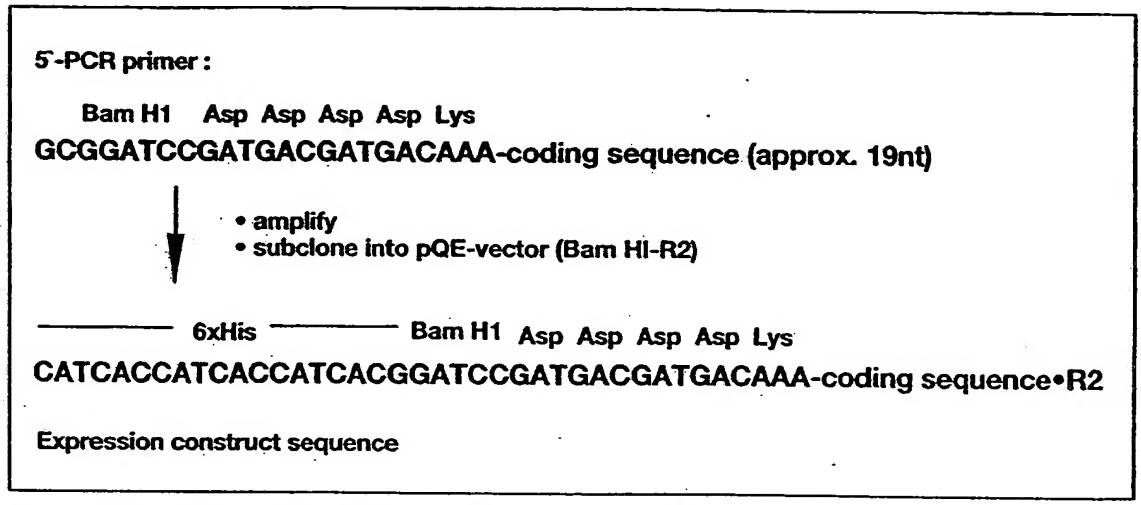


Figure 6: Insertion of enterokinase cleavage site by PCR.

Section B - Procedures

The gene or protein sequence to be expressed is subcloned into the appropriate pQE vector in the same reading frame as the 6xHis affinity tag. The pQE expression construct is then transformed into M15[pREP4] or SG13009 [pREP4] host strain carrying the pREP4 repressor plasmid. Transformants are selected on plates containing both ampicillin and kanamycin.

Cultures are grown in the presence of ampicillin and kanamycin. After they reach a certain cell density, they are induced by the addition of IPTG. The cells are allowed to express the recombinant protein for some hours before harvesting.

The cells are lysed under either non-denaturing or denaturing conditions, and the cleared lysate is loaded onto the Ni-NTA column, or adsorbed to the resin in a batch procedure. After a number of washing steps, the pure protein is eluted, ready for use without further modification.

In some cases, it is advisable to first lyse cells under non-denaturing conditions and purify native proteins from the supernatant. The protein from inclusion bodies can then be purified under denaturing conditions.

IV. Preparation of Expression Constructs

1. General Considerations

Most of the pQE vectors contain a polylinker with a number of potential cloning sites and are supplied in three reading frames to simplify the subcloning of coding fragments. The control regions of all the pQE vectors are shown in Fig. 7. The variable region is different for each pQE vector, and is shown in detail in the following figures and Appendix D. Restriction maps for prototype pQE vectors can be found in Appendix E.

In general, it is only necessary to locate the appropriate restriction site, identify the correct reading frame, and subclone the fragment directly. If there is no appropriate restriction site or if it is desirable to minimize the number of extra codons, or to optimize the construct in some other way, then more complicated manipulations may become necessary. The ends of coding fragments can be modified by PCR (see Fig. 8), by *in vitro* mutagenesis, or by the addition of linkers. It is recommended that those unfamiliar with these procedures consult a practical manual, such as *Molecular Cloning* (Sambrook *et al.*, 1989).

Expression vectors such as the pQE plasmids do not provide for color selection of plasmids that contain inserts after ligation and transformation. Care should be taken to ensure that vectors are digested to completion before subcloning, and dephosphorylated

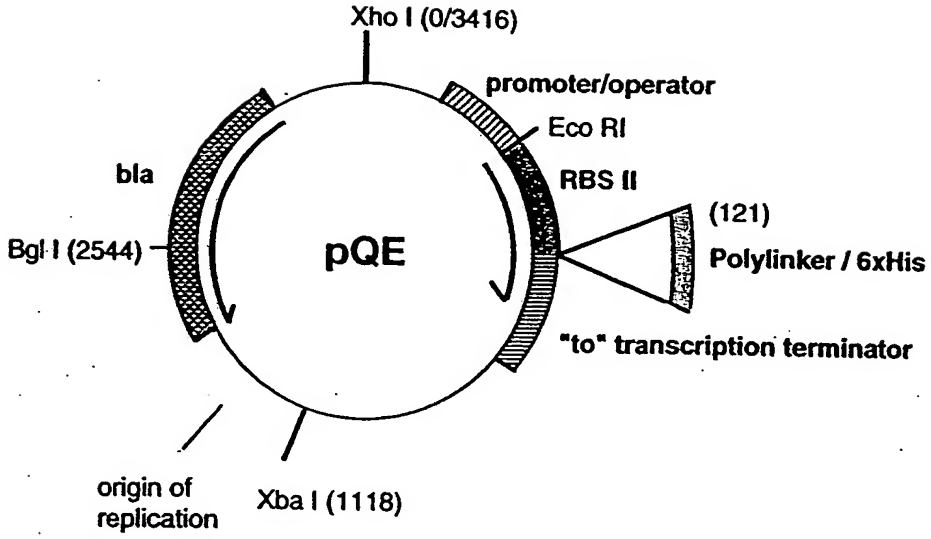


Figure 7: Control regions of QIAexpress pQE-vectors.

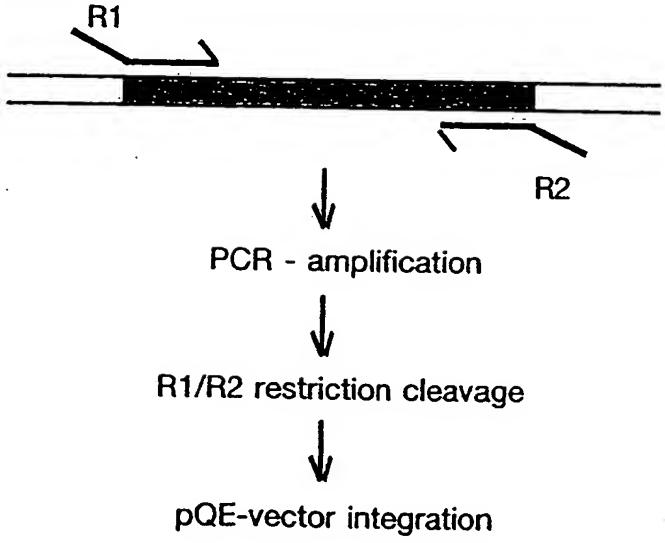


Figure 8: Subcloning into pQE-vectors by PCR.

where necessary. The efficiency of the subcloning should always be monitored by the transformation of unligated and self-ligated vector controls. A procedure for the transformation of M15[pREP4] can be found in Protocol 1.

Transformants may be screened for correct insertion of the coding fragment by restriction analysis of the DNA, sequencing of the cloning junctions, or by preparing small scale expression cultures. We prefer screening directly for expression because it allows the simultaneous screening of transformants for the correct coding fragment, expression levels, and in-frame translation of the 6xHis tag. A procedure for direct screening of mini expression cultures can be found in Protocol 2. The primers used for sequencing the cloning junction can be found in Appendix F.

2. Type IV Constructs

The pQE vectors for these constructs have the 6xHis tag 5′ to the polylinker (Fig. 10a, b). The appropriate vector need only be digested with the necessary restriction enzyme(s), the linear form isolated, and the coding region ligated to it in-frame. If only one restriction site is used, we recommend that the ends of the vector be dephosphorylated to prevent religation.

It is best to remove the start codon and any upstream control regions from the fragment that is to be inserted into these vectors. Internal starts from control sequences provided by the inserted fragment will result in expressed proteins that do not contain the 6xHis tag, and cannot be isolated.

The authentic stop codons can be retained in the inserted fragment, but they are not necessary as the pQE vectors provide translational stop codons in all three reading frames 3′ to the polylinker.

3. Type III Constructs

Type III constructs place the 6xHis affinity tag at the C-terminus of the protein. They require that the insert is in frame with both the 5′ start codon and the 3′ sequences coding for the 6xHis tag. This is achieved by preparing 5′ and 3′ 'acceptor arms' in the appropriate reading frames, and then recreating the expression construct by a 3-arm ligation between these acceptors and the coding fragment (Fig. 11a). The multi-cloning site vectors pQE-50, -51 and -52 provide the 5′ acceptor arms, and the vectors pQE-16, -17 and -18 provide the 3′ acceptor arms (Fig. 11b).

The appropriate vectors are digested first with the chosen restriction enzymes for ligation to the coding fragment. They are then digested with the enzyme *Bgl* I, and the correct

fragments prepared on an agarose gel. If necessary (e.g. when the two ends of the insert are compatible) a dephosphorylation step should be introduced before the *Bgl* I digestion. The enzyme *Bgl* I has an asymmetrical overhang which ligates very efficiently and only 'head-to-tail', allowing the 3-arm ligation to proceed almost as efficiently as a 2-arm ligation. The unique *Xba* I site may also be used for the second digestion, but will not religate so efficiently.

As with Type IV constructs, it is also recommended that the native start codon and upstream activation sequences are removed from the coding fragment. The control regions in the pQE vectors are optimized for expression in *E. coli* and the incorporation of alternate control regions may reduce their efficiency. Since the 6xHis affinity tag sequences are located 3' to the inserted fragment, any in-frame translational stop codon should also be removed. For isolation of DNA fragments from agarose gels for subsequent ligation reactions we recommend the use of QIAEX Gel Extraction Kit (#20020).

4. Type ATG Constructs

Type ATG constructs allow the expressed protein to start with the authentic ATG codon, but maintain the optimized control regions from the pQE vector. They have the 6xHis tag at the C-terminus of the protein, and are thus Type III constructs. The pQE vectors for Type ATG constructs are shown in Fig. 12a, b.

In order to recreate the authentic ATG codon, the sequence around that codon must be modified to form either an *Nco* I site (for pQE-60) or an *Sph* I site (for pQE-70). When the insert is ligated to the appropriate vector, it's ATG codon will replace the vector ATG codon. The sequence at the 3' end of the insert must be ligated in frame with the 6xHis tag in the vector. This requires the formation of a *Bam* HI site (or other restriction site that creates a GATC overhang) in the correct reading frame, 5' to any stop codons. Insert sequences can be easily modified to fit these requirements using PCR or other methods of *in vitro* mutagenesis.

5. Type II Constructs

The pQE vectors for creating Type II constructs (Fig 13a, b) place the 6xHis tag and the sequence coding for mouse DHFR 5' to the polylinker. The constructs are created in the same way as Type IV constructs, by simply opening the appropriate pQE vector at the required restriction site(s) and inserting the fragment in frame with the DHFR sequence. All the considerations mentioned in Section IV.2 apply equally to the preparation of Type II constructs.

6. SRF Vectors

Single reading frame vectors (Fig. 9) are available for the preparation of both Type IV (pQE-8) and Type III (pQE-12) constructs. As the name implies, these vectors are supplied in only one reading frame, so the reading frame of the insert must be adjusted accordingly. Adjustments to the reading frame of insert sequences, and creation of new restriction sites, is achieved through PCR or other forms of *in vitro* mutagenesis.

7. Propagation of pQE Vectors and Constructs

The QIA*express* pQE vectors and constructs can be maintained and propagated in any *E. coli* strain that is ampicillin sensitive and carries either the *lacI*^{q} gene or the pREP4 repressor plasmid. We recommend that pQE plasmids be propagated in *lacI*^{q} strains to avoid any confusion with the pREP4 plasmid. The use of strains that carry only the *lacI* gene, which gives 10-fold lower levels of *lac* repressor, is not recommended.

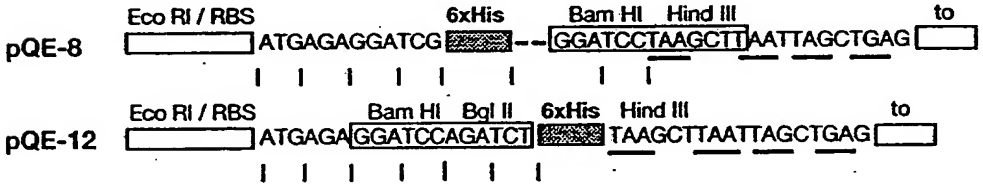


Figure 9: QIAexpress SRF vectors.

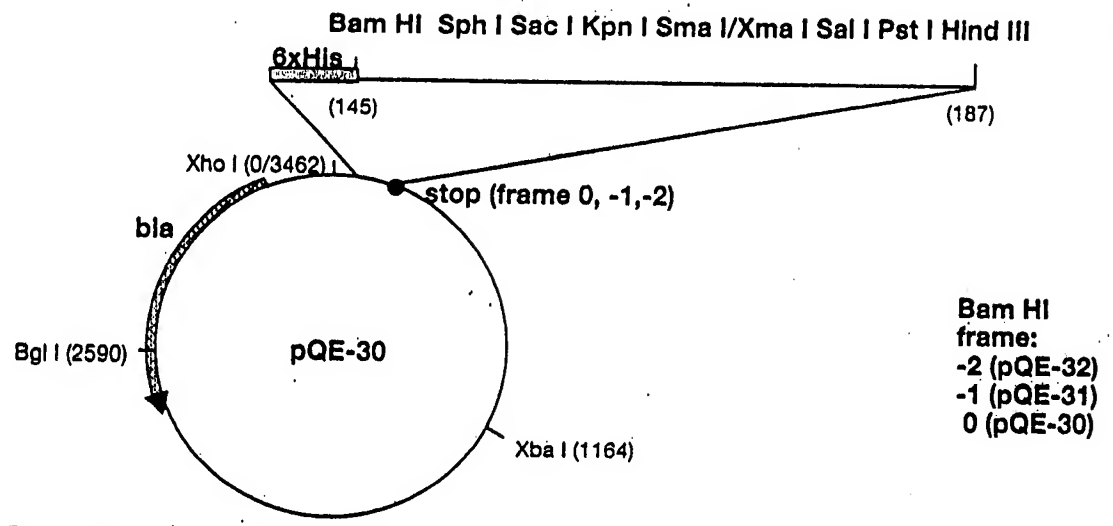


Figure 10a: Cloning into Type IV vectors.

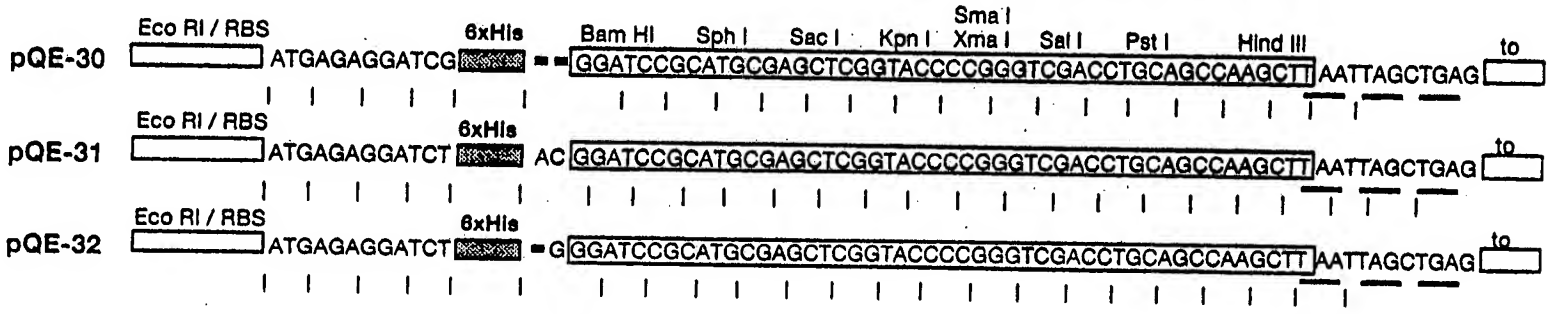


Figure 10b: Multi-cloning region of Type IV vectors.

QIAexpress Type III Constructs

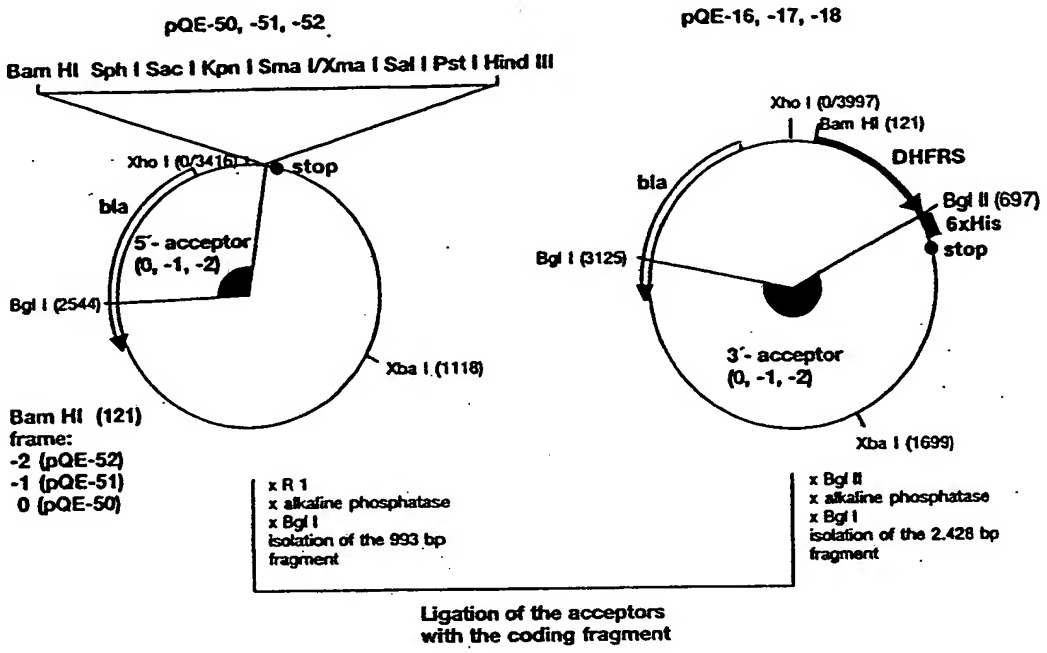


Figure 11a: Cloning into Type III vectors.

QIAexpress Type ATG Constructs

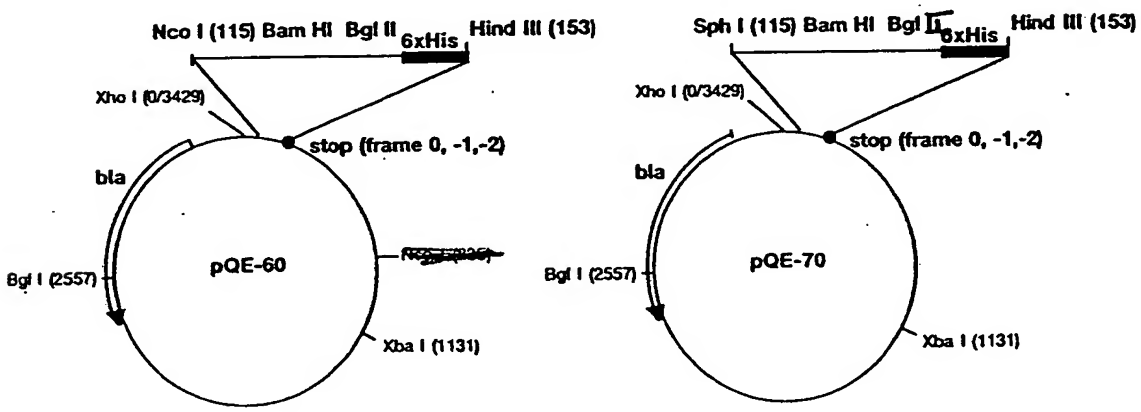


Figure 12a: Cloning into Type ATG vectors.

QIAexpress Type III Constucts

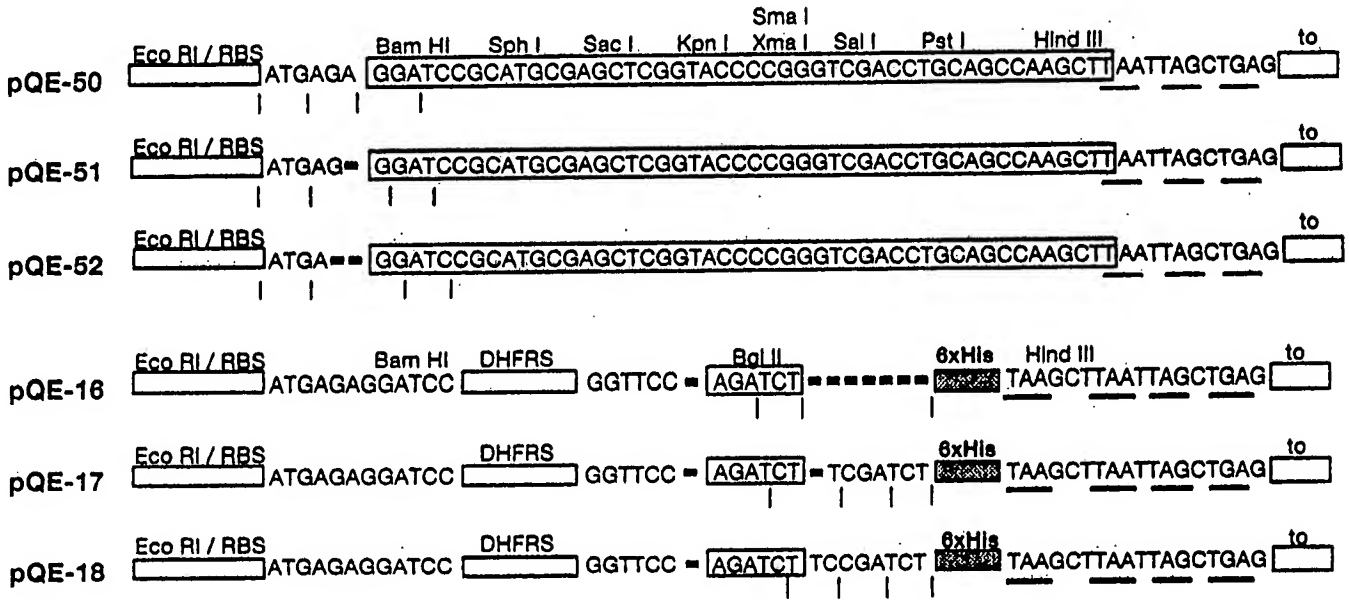


Figure 11b: Multi-cloning region of Type III vectors.

QIAexpress Type ATG Constructs

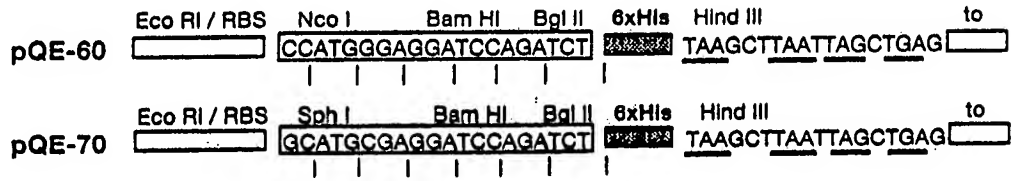


Figure 12b: Multi-cloning region of Type ATG vectors.

QIAexpress Type II Constructs

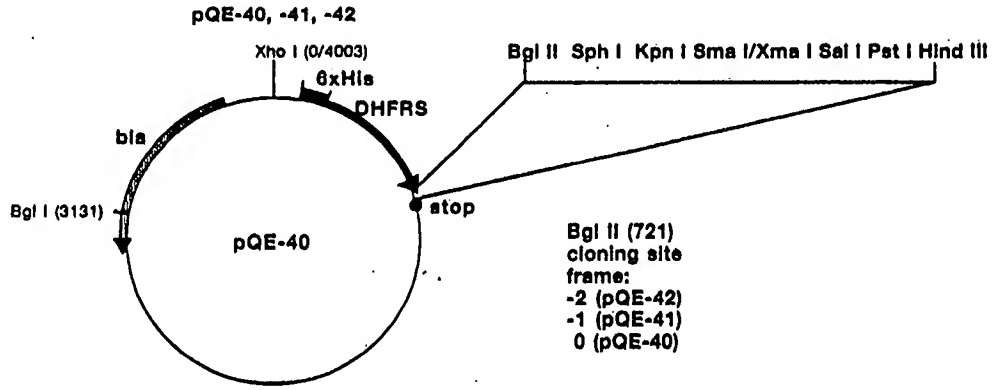


Figure 13a: Cloning into Type II vectors.

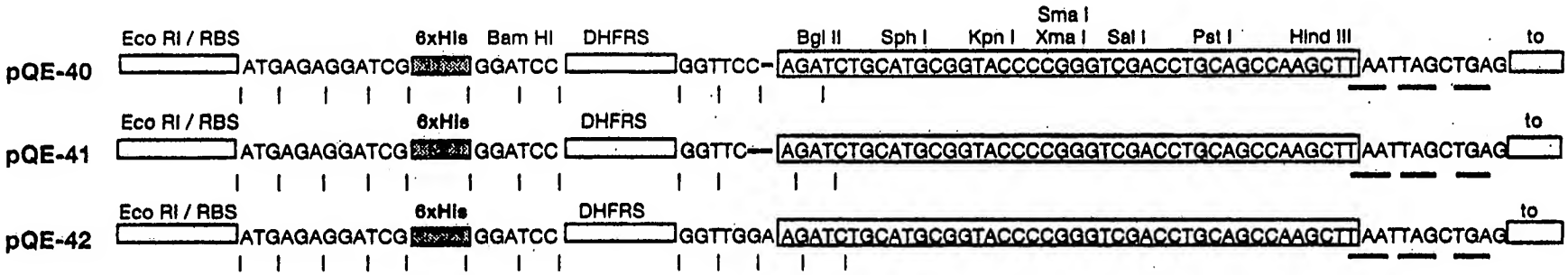


Figure 13b: Multi-cloning region of Type II vectors.

Protocol 1

Preparation of Competent M15 [pREP4] Cells and Transformation of Plasmids

1. Remove a trace of M15[pREP4] cells from the vial with a sterile toothpick and streak out on an LB/25 µg/ml kanamycin plate.

2. Incubate at 37°C overnight.

3. Pick a single colony and inoculate 1 ml of LB/25 µg/ml kanamycin. Grow to mid log phase and inoculate 100 ml of LB/25 µg/ml kanamycin in a 250 ml flask, and shake at 37°C until an A_{600} of 0.5 is achieved.

4. Centrifuge in a sterile tube (round bottom) at low speed (5 min, 2000 rpm, 4°C).

5. Discard the supernatant carefully.

6. Resuspend the cells gently in cold (4°C) TFB1 buffer (30 ml/100 ml culture) and keep on ice for an additional 90 minutes.
(TFB1: 100 mM RbCl, 50 mM $MnCl_2$, 30 mM KAc, 10 mM $CaCl_2$, 15% glycerol, pH 5.8, sterile-filtered.)

7. Centrifuge (5 min, 2000 rpm, 4°C).

8. Discard the supernatant carefully. Always keep the cells on ice.

9. Resuspend the cells carefully in ice cold TFB2 buffer (4 ml/100 ml culture).
(TFB2: 10 mM MOPS, 10 mM RbCl, 75 mM $CaCl_2$, 15% glycerol, pH 8.0, autoclaved.)

10. Prepare aliquots of 500 µl in sterile microfuge tubes and snap freeze in liquid nitrogen. Store at -70°C.

Transformation

1. Transfer the ligation mix (20 µl, or less) into a cold 5 ml sterile plastic tube.

2. Take an aliquot of frozen M15[pREP4] competent cells and thaw on ice.

3. Gently resuspend the cells and carefully transfer 125 µl of cell suspension to the plastic tube with the ligation mix. Mix carefully and keep on ice for 20 min.

4. Transfer the tube to a 42°C waterbath for 90 sec.

5. Add 500 µl Psi-broth (LB medium, 4 mM $MgSO_4$, 10 mM KCl) and shake at 250 rpm for 90 min at 37°C.

6. Plate 50 µl and 450 µl on LB-agar plates containing 25 µg/ml kanamycin and 100 µg/ml ampicillin (or other selective agent). Incubate at 37°C overnight.

Positive control:
Transform with 1 ng of control plasmid in 20 µl of TE. Plate 1:500, 1:100 and 1:10 dilutions of the transformation mix (diluted in Psi-broth). The cells should yield approximately 1 x 10^6 transformants per µg of plasmid.

Negative control:
Transform with 20 µl of TE. Plate entire transformation on one plate.

V. Expression of Recombinant Proteins

With careful choice of host strains, vectors and growth conditions, most recombinant proteins can be expressed to high levels in *E. coli*. However, some proteins are very toxic to cells, and are expressed poorly. Other proteins are very unstable and subject to degradation. The QIA*express* system is optimized to give high expression levels, but in the final analysis, the conditions for optimal expression of individual proteins must be determined empirically on a case-by-case basis. It is strongly recommended that the optimal growth and expression conditions for the protein of interest are established with small-scale cultures before large-scale protein purification is attempted. A procedure for this can be found in Protocol 3.

Expression of DHFR (19 kD) from the control plasmid, pQE-16, is strongly recommended. It serves as a control for both expression and purification. The following section provides some advice about the expression of difficult proteins. (Please note that DHFR-6xHis runs at 26 kD on SDS-PAGE gels.)

1. Hydrophobic Regions

The presence of hydrophobic regions in recombinant proteins often has a toxic effect on the host cells, probably due to association of the protein with vital membrane systems. Such regions, particularly signal peptides and transmembrane sequences, should be removed from the recombinant protein if they are not of specific interest.

2. Toxic Genes and Proteins

Some genes are only mildly toxic, and only display poor expression after they have been growing on plates or in culture for some days. Such problems can usually be overcome by expressing proteins in freshly transformed cells. More toxic genes are generally characterized by the presence of very few transformants in comparison to the parent vector. The transformants that do arise often have deletions and mutations, and should be carefully checked. These problems can sometimes be overcome by growing the cells in the following way. The cultures should be grown with high levels of ampicillin (200 µg/ml), and supplemented with 2% glucose to maximize the repression of expression. Growing at lower temperatures (30°C) is also sometimes useful. Overnight starter cultures should be avoided. A single colony from a fresh plate should be inoculated into a 2 ml starter culture which should be grown for 2-3 hours, until it is in mid-log phase. This should be inoculated into 400 ml of fresh media, and the culture grown to an A_{600} of approximately 0.5 before induction. This procedure may prevent the death of cells that are producing low levels of protein during growth.

3. Unstable Proteins

Some proteins, particularly those that are short, are not stable in *E. coli*, and may be broken down rapidly by cellular proteases. This may be overcome by reducing the growth temperature; inducing for a shorter period of time; or changing to a host strain deficient in one or more proteases. Short proteins and peptides may become more stable when expressed as a fusion with DHFR (see Section III. 3).

If the protein is degraded during the purification process, it may be necessary to use one or more protease inhibitors such as PMSF.

4. Maintaining the Expression Construct

Low expression levels are sometimes the result of poor plasmid maintenance in the cells. Ampicillin is a labile antibiotic, and is rapidly used up in growing cultures. It is important to check plasmid levels by plating cells from the expressing culture on plates plus and minus ampicillin. If the stability of the expression construct is a problem, the cultures should be grown in the presence of 200 µg/ml ampicillin and the level maintained by addition of extra ampicillin during long growth periods. Alternatively, the cultures may be grown in the presence of carbenicillin, a more stable semisynthetic form of ampicillin, at 50 µg/ml. The repressor plasmid is maintained in the presence of kanamycin at 25 µg/ml.

5. Low Expression Levels

Low expression levels can occur for any one of the above reasons. It is important to establish whether expression is low because the protein is toxic, unstable, or because the expression construct is not being maintained in the cells during growth.

The nature of the 5′ end of the gene being expressed should be checked (see Section III.6), and modified if necessary. It is also worth trying a number of different growth media and host strains.

6. High Expression Levels and Insoluble Proteins

The QIA*express* system provides for very efficient expression of recombinant proteins, however sometimes these high expression levels can create problems with the solubility of the expressed proteins. Most insoluble proteins form inclusion bodies in *E. coli* which can be easily solubilized in denaturants such as 6 M guanidine hydrochloride or 8 M urea. These denaturants, as well as many detergents, do not effect the affinity of the 6xHis tag for the Ni-NTA resin, so purification of insoluble proteins is not a problem with QIA*express*. The pure proteins can then be renatured if necessary before use.
Please see Section IV.6. for more information on protein refolding.

The presence of inclusion bodies does not necessarily mean that non-denaturing purification strategies are not possible. Native proteins often remain soluble in the cytoplasm, and may be abundant enough to provide reasonable yields. In some cases, however, it is desirable to keep the protein soluble at all times, and this generally requires that the expression levels be reduced in some way. It may be sufficient to simply change the host strain that is used, since some strains tolerate some proteins better than others, and allow higher levels of expression before forming inclusion bodies. The growth temperature often directly effects both expression levels and protein solubility, and lower temperatures will often keep the expressed protein soluble. Reduction of IPTG levels during induction can also reduce expression, or the culture can be grown to a higher cell density before induction, and the expression period kept to a minimum. Finally, many proteins require metal co-factors in order to remain soluble, and metal supplements in the media may be helpful. If the metal requirements of the protein are not known, it will be necessary to try a number of different supplements. Please note that metal supplements may increase the activity of metal requiring proteases.

7. Culture Media

The media of choice for the growth of M15 cells containing a pQE expression plasmid and the pREP4 repressor plasmid is LB-medium and its modifications, 2x YT or Super Broth, containing 100 µg/ml ampicillin and 25 µg/ml kanamycin. We suggest that expression should be tried in all three media in parallel, and a time course taken of expression after induction (See Protocol 2). There are often striking differences noted between the level of expression in different media at different times.

Inclusion of 2% glucose in the culture media may be useful when expressing toxic proteins, in order to maximize repression of transcription.

8. Small-scale Expression Cultures

It is always best to try small-scale expression and purification of the recombinant protein before moving into a large scale experiment. Expression levels vary between different colonies of transformed cells, and preparing a number of mini cultures allows selection of the optimal expressers.

It is also a very rapid way to judge the effects of varied growth condition on expression levels and solubility of recombinant proteins.

The number of colonies to be screened will depend on whether these are primary transformants, or transformants that have already been screened for the correct insertion of the coding fragment into the expression vector. If primary transformants are being

screened, then the number will depend on the efficiency of the ligation (as estimated from comparison with the vector controls), and whether the cloning was directional. If the transformants are known to contain the coding fragment in the correct orientation, it should be necessary to screen only a few colonies.

It is best to screen a number of transformants for the ability to express a protein bearing the 6xHis tag, and then choose only one on which to optimize the culture conditions to maximize expression.

Protocol 2

Rapid Screening of Small-scale Expression Cultures

The following is a basic protocol for the expression and screening of small cultures. It contains no stringent washing steps, and therefore cannot be expected to give highly pure product, but it will show whether there is a protein being expressed with an affinity tag, and the level of expression. Purification is performed under denaturing conditions, which will lead to the isolation of any tagged protein, independent of its location and constitution within the cell.

When analyzing a time course of the expression characteristics, it is best to begin with a 10 ml culture, and to take 1 ml samples at t = 0, 1, 2, 3 and 4 hours after induction.

The culture media should contain ampicillin at 100 µg/ml and kanamycin at 25 µg/ml.

1. Pick single colonies of transformants into 1.5 ml of culture media containing both ampicillin and kanamycin. Also inoculate one 1.5 ml culture with a colony transformed with the control plasmid, pQE-16, which expresses DHFR. Inoculate one extra culture to serve as an uninduced control. Grow the cultures overnight.

2. Inoculate 1.5 ml of prewarmed media (+antibiotics) with 500 µl of overnight cultures, and grow at 37°C for 30 min., with vigorous shaking, until the A_{600} is 0.7 - 0.9.
   The short second growth, from an aliquot of the saturated culture, is to ensure that all cultures are grown to a similar cell density before induction. If expression levels are being closely compared, it is advisable to measure the A_{600} to ensure uniform cell density.
   If 10 ml cultures are being grown to prepare a time course of expression, inoculate 8.75 ml of medium with 1.25 ml of saturated culture, and grow for 1 hr.

3. Induce expression by adding IPTG to a final concentration of 2 mM. Do not add IPTG to the culture which will serve as an uninduced control.
   If a time course of expression is being taken, the t = 0 sample serves as the uninduced control.

4. Grow the cultures for an additional 3 - 5 hr, and transfer 1 ml to a micro-centrifuge tube. Harvest the cells by centrifugation for 3 min at 3000 rpm, and discard supernatants.
   Cells may also be harvested at 15,000 rpm in the microfuge, but in this case should be spun for only 20 seconds. If a time course of expression is being

prepared, take 1 ml samples at hourly intervals, collect the cell pellet and store it at -20°C until all the samples are ready for processing.

5. Resuspend cells in 200 µl Buffer B (8 M urea, 0.1 M Na-phosphate, 0.01M Tris/HCl pH 8.0). Lyse cells by gently vortexing, taking care to avoid frothing.
   The solution should become translucent when lysis is complete. Most proteins are soluble in Buffer B.

6. Centrifuge the lysate for 10 min at 15,000 rpm (in a microcentrifuge) to pellet the cellular debris, and transfer the supernatant to a fresh tube.

7. Add 30 - 50 µl of a 50% slurry of Ni-NTA resin to each tube, and mix gently for 30 min at room temperature.

8. Centrifuge 10 sec at 15,000xg to pellet the resin, transfer 10 µl of the supernatant to a fresh tube, and discard the remaining supernatant. Store the supernatant samples on ice.
   The supernatant samples will contain any proteins which have not bound to the resin.

9. Wash the resin 3x with 1 ml of Buffer C (8 M urea, 0.1 M Na-phosphate, 0.01M Tris/HCl pH 6.3).

10. Add 20 µl of Buffer C/100 mM EDTA to each tube. Incubate at room temperature for 2 min with gentle mixing.
    The EDTA chelates the Ni^{2+} ions from the NTA resin, and elutes the protein.

11. Centrifuge for 10 sec at 15,000 rpm and carefully remove 20 µl of the supernatant to a fresh tube.

12. Add 5 µl of 5x PAGE sample buffer (15% ß-ME; 15% SDS; 1.5% bromophenol blue; 50% glycerol) to all samples, including the unbound fractions from step 8, and boil for 7 min at 95°C.

13. Analyze samples on 12.5% polyacrylamide gels containing 0.2% SDS (Takacs, 1979). Visualize proteins by staining with Coomassie blue.

Protocol 3

Growing Large-scale Expression Cultures

This procedure has been optimized for the expression of DHFR-6xHis in strain M15[pREP4], and works well for many proteins. It yields approximately 50 mg of DHFR-6xHis per liter of culture.

1. Inoculate 10-20 ml of LB-broth containing 100 µg/ml ampicillin and 25 µg/ml kanamycin with a single colony containing the expression plasmid, or with 100 µl of glycerol stock. Grow at 37°C overnight, with vigorous shaking.

2. Inoculate a large culture (LB, 100 µg/ml ampicillin, 25 µg/ml kanamycin) 1:50 with the uninduced overnight culture. Grow at 37°C with vigorous shaking until the A_{600} reaches 0.7 - 0.9.

3. Add IPTG to a final concentration of 1-2 mM, and continue to grow the culture at 37°C for 5 hours.

4. Harvest the cells by centrifugation at 4000xg for 10 min.

5. Store cell pellet at -70°C if desired, or process immediately according to one of the protocols found in Section VI of this manual.

VI. Purification on Ni-NTA-Resin

While many proteins remain soluble during expression and can be purified in their native form under non-denaturing conditions on Ni-NTA resin, many others will form insoluble precipitates. Since almost all of these proteins are soluble in 6 M guanidine hydrochloride, Ni-NTA chromatography and the 6xHis tag provide a universal system for the purification of recombinant proteins.

This manual provides procedures for purification of proteins from *E. coli* under both denaturing and non-denaturing conditions. While each procedure will work very well for most proteins, some modifications may be necessary if host systems other than *E. coli* are used. The tremendous purification power of the QIA*express* system will be enhanced if the conditions are optimized for each individual protein. The discussion below will aid in this process.

1. Checking for Protein Solubility and Cellular Location

Before deciding on a purification strategy, it is important to determine whether the protein is soluble in the cytoplasm, located in cytoplasmic inclusion bodies, or secreted into the periplasmic space. Many proteins form inclusion bodies when they are expressed at high levels in bacteria, while others are well tolerated by the cell and remain in the cytoplasm in their native configuration.

Proteins that contain secretion signals may be secreted into the periplasmic space, but it depends on the nature of both the signal and the recombinant protein. A procedure for determining the location and solubility of the expressed protein can be found in Protocol 4.

2. Purification under Native or Denaturing Conditions

The decision whether to purify the tagged proteins under denaturing or non-denaturing conditions depends on both the location of the protein, and the accessibility of the 6xHis affinity tag. Proteins that remain soluble in the cytoplasm, or are secreted into the periplasmic space, can generally be purified under non-denaturing conditions (but please note the exception below).

If the protein is insoluble, or located in inclusion bodies, then it must generally be solubilized by denaturation before it can be purified. Some proteins, however, may be solubilized by the addition of detergents, and it is worth experimenting with different solubilization techniques if it is important to retain the native configuration of the protein. Many proteins that form inclusion bodies are also present at some level in the cytoplasm, and may be efficiently purified in their native form, even at these very low levels, on Ni-NTA resin.

In rare cases the 6xHis tag is hidden by the tertiary structure of the native protein, so that soluble proteins require some level of denaturation before they can be purified on Ni-NTA. If denaturation of the protein is undesirable, the problem is usually solved by moving the 6xHis tag to the opposite terminus of the protein.

Proteins that have been purified under denaturing conditions can either be used directly, refolded in dilute solution, or refolded on the Ni-NTA column. Please refer to Section VI.6 for more information. Proteins bound to the column in this way may serve as an affinity matrix for the isolation of associated proteins (Bugge *et al.*, 1992).

3. Batch or Column Purification

Proteins may be purified on Ni-NTA resin in either a batch or a column procedure. Both procedures are equally efficient, and the choice is really up to the user. The batch procedure entails binding the protein to the Ni-NTA resin in solution, and then packing the protein/resin complex into a column for the washing and elution steps. This may promote more efficient binding of the tagged protein, and reduce the amount of debris that is loaded onto the column. Batch binding for an extended time is recommended when purifying very dilute proteins. Please refer to Section VI.5.

In the column procedure, the Ni-NTA column is packed and washed and the cell lysate is applied slowly to the column, at approximately 3 - 4 column volumes per hour.

4. Removal of Contaminating Proteins

Background contamination can arise from proteins that contain neighboring histidines, and thus have some affinity for the resin; proteins that co-purify because they are linked to the 6xHis-tagged protein by disulfide bonds; proteins that associate non-specifically with the tagged protein; and nucleic acids that associate with the tagged protein. All of these contaminants can be easily removed by washing the resin under the appropriate conditions, as described in the protocols.

Proteins that contain neighboring histidines are not common in bacteria, but are quite abundant in mammalian cells. These proteins bind to the resin much more weakly than proteins with a 6xHis tag, and can be easily washed away, even when they are much more abundant than the tagged protein (Janknecht *et al.*, 1991). The addition of 10 mM ß-ME to the loading buffer will reduce background due to cross-linked proteins. This is important when purifying proteins which contain cysteine residues. Do not use DTT or DTE, which will reduce the Ni^{2+} ions.

Proteins which are associating with the tagged protein or the resin due to non-specific interactions, and nucleic acids that co-purify, can be removed by washing with low levels of non-ionic detergent (0.1-1% Triton® X-100 or Tween®-20); increasing the salt concentration (up to 1 M NaCl); or including ethanol or glycerol (up to 30%) to reduce hydrophobic interactions. The optimum levels of any of these reagents should be determined empirically for different proteins.

5. Optimizing Conditions for Binding, Washing and Elution

Removal of background proteins and elution of tagged proteins from the column may be achieved by either lowering the pH in order to protonate the histidine residues, or by the addition of imidazole, which competes with the tagged proteins for binding sites on the Ni-NTA resin. While both methods are equally effective, the imidazole is milder, and is recommended in cases where the protein would be damaged by a reduction in pH.

In bacterial expression systems, it is rarely necessary to wash the bound protein under very stringent conditions, since proteins are expressed to high levels and the background is low.

In eukaryotic systems however, or under non-denaturing conditions where proteins are more likely to present neighboring histidines, it may be necessary to increase the stringency of the washing considerably. This can be done by gradually decreasing the pH of the wash buffer, or by slowly increasing the concentration of imidazole. The pH or imidazole concentration which can be tolerated before elution begins will vary slightly for each protein, and must be determined empirically.

In situations where the tagged protein is very dilute, and the background is likely to be high, it is also useful to bind the 6xHis tagged protein to the resin under conditions in which the background proteins do not compete for the binding sites, i.e., at a slightly reduced pH or in the presence of low levels of imidazole.

Likewise, the purification process will be optimized if the amount of tagged protein is closely matched to the capacity of the resin used i.e. if the amount of resin is minimized (H. Stunnenberg, personal communication). Since the 6xHis-tagged protein has a higher affinity for the Ni-NTA resin than do the background proteins, if it can fill all the available binding sites, very few background proteins will be retained on the resin. This effect is enhanced by performing the binding in a batch procedure and allowing it to proceed for about an hour. This forces the equilibrium towards binding of the 6xHis-tagged proteins and exclusion of the non-tagged proteins which bind more weakly.

6. Protein Refolding

Each denatured protein needs to be refolded according to a specially optimized protocol. Refolding is generally carried out by gradual dilution of the denaturing agents, together with careful reformation of the disulfide bridges. While there are few hard and fast rules about protein refolding, the following guidelines may prove helpful.

As a general rule, protein refolding should take place slowly and in dilute solution to avoid the formation of insoluble aggregates. It should be carried out at a redox potential which is close to the equilibrium of Cys-SH and Cys-S-S-Cys. Disulfide bridges then only become stabilized if they are trapped in a strong and correct tertiary structure. The addition of detergents, salt and alcohol to the refolding buffer may aid in keeping the refolded proteins soluble.

Refolding is generally carried out by step-wise dilution of denaturants in a dialysis procedure. One folding buffer system used successfully in zinc finger protein refolding is: 1 M urea, 0.05 M Tris/HCl, 0.005% Tween®-80, 2 mM reduced glutathione, 0.02 mM oxidized glutathione, pH 8.0, and stirring at 4°C for 30 hours (see Jaenicke, R. & Rudolph, R. (1991)).

Many proteins which are insoluble when refolded in solution can be successfully refolded while immobilized on the Ni-NTA column. It may be that immobilizing one end of the protein during renaturation prevents the formation of misfolded aggregates. The recommended conditions are as follows: Renature using a linear 6 M - 1 M urea gradient in 500 mM NaCl, 20% glycerol, Tris/HCl pH 7.4, containing protease inhibitors. The gradient should be FPLC® mediated to ensure linearity (conventional gradient makers often make nonlinear gradients), and the renaturation should take place over a period of 1.5 hours. After renaturation the proteins can be eluted by the addition of 250 mM imidazole (E. Weston, personal communication).

7. Limitations

Do not use strong reducing agents such as DTT or DTE on the column, as they will reduce the Ni^{2+} ions and cause them to elute from the resin. In most situations, ß-ME can be used at levels up to 10 mM, but even these low levels might cause problems occasionally when the protein itself has a strongly reducing nature. Use any reducing agent with care, and if in doubt, test it out on a small amount of Ni-NTA resin first.

Strong chelating agents will chelate the Ni^{2+}, and cause it to leach from the NTA resin. Do not use EDTA, or EGTA, or any other chelating agents on the column. Most ionic detergents also interfere with binding to the Ni-NTA resin. While some ionic detergents, such as sarkosyl, have be used successfully at low levels (0.01%), they are not recommended, and should be replaced with non-ionic detergents.

Protocol 4

Checking for Cytoplasmic or Periplasmic Location

1. Grow a 100 ml culture and induce according to the standard procedure (Protocol 3). Take a 1 ml sample immediately before induction (**uninduced control**), pellet cells and resuspend in 50 µl of SDS-PAGE sample buffer. Freeze until use. Before harvesting, collect a second 0.5 ml sample (**induced control**), pellet cells and resuspend in 100 µl of SDS-PAGE sample buffer. Freeze until use.

2. Divide the culture into two aliquots. Harvest the cells by centrifugation at 4000xg for 10 min.

A. Check for cytosolic localization

1A. Resuspend one pellet in 5 ml of sonication buffer (50 mM Na-phosphate pH 7.8, 300 mM NaCl).

2A. Freeze sample in dry ice/ethanol, and thaw in cold water.

3A. Sonicate briefly to lyse cells (avoid frothing).

4A. Centrifuge at 10,000xg for 20 min. Decant the supernatant (**crude extract A**, soluble protein) and save on ice.

5A. Resuspend the pellet in 5 ml sonication buffer. This is a suspension of the insoluble matter (**crude extract B**, insoluble protein).

B. Check for periplasmic localization

1B. Resuspend the other pellet in 10 ml 30 mM Tris/HCl, 20% sucrose, pH 8.0.

2B. Add EDTA to 1 mM, and incubate at room temperature for 5 - 10 min, with shaking or stirring.

3B. Centrifuge at 8000xg at 4°C for 10 min, remove all the supernatant, and resuspend the pellet in 10 ml ice-cold 5 mM $MgSO_4$.

4B. Shake or stir for 10 min in an ice/water bath.

5B. Centrifuge at 8000xg at 4°C for 10 min. The supernatant is the osmotic shock fluid (**extract C**, periplasmic extract).

SDS-PAGE analysis:
Add 5 µl of 2x SDS-PAGE sample buffer to 5 µl of crude extracts A&B and 10 µl of 2x SDS-PAGE sample buffer to 10 µl of extract C. Boil samples, along with the uninduced and induced cell samples, for 5 min. Microcentrifuge for 1 min. Load 20 µl of the uninduced and induced cell samples, and all of the extract samples, on a 10% SDS-PAGE gel. Optional: Run an identical gel using 1:5 dilutions of the same samples, in SDS-sample buffer.

Note

a. If the fusion protein is in the periplasmic fraction (extract C), a pilot experiment (e.g. varying induction conditions) to optimize export of the protein is recommended.

b. If the fusion protein is in the insoluble matter (extract B), ensure that the cells are completely lysed. If it is still insoluble, try extracting the pellet with 0.25% Tween®-20, 0.1 mM EGTA a few times. Often the pellet is not truly insoluble but just associated with the the membrane fragments in the cell pellet. If the protein is truly insoluble under these conditions, try solubilizing and purifying it under denaturing conditions.

[1]PMSF is highly toxic and must be handled with care.

8. Purification of Native Proteins under Non-denaturing Conditions

Before purifying proteins under non-denaturing conditions, it is important to check that the protein is soluble (Protocol 4). Even if most of the protein is insoluble, it is possible to purify any soluble material on Ni-NTA resin. There is often soluble material that has not been incorporated into inclusion bodies remaining in the cytoplasm.

General Considerations

Unstable proteins may be subject to degradation during cell harvest and lysis in the absence of strong denaturing agents such as guanidine hydrochloride. It is best to work quickly and to keep the cells at 0 - 4°C at all times. Addition of PMSF[1] (0.1 mM) and other protease inhibitors may be helpful on a case to case basis, but their potential effect on the recombinant protein must be taken into consideration. Many irreversibly modify proteins, thereby interfering with their biological activity.

Protocol 5

Native Purification of Cytoplasmic Proteins

1. Grow and induce a 1 l culture as described in Protocol 3.

2 Harvest the cells by centrifugation at 4000xg for 20 min. Resuspend pellet in Sonication Buffer (50 mM Na-phosphate pH 8.0, 300 mM NaCl) at 2 - 5 volumes per gram of wet weight.

3. Freeze sample in dry ice/ethanol (or store overnight at -20°C), and thaw in cold water. Alternatively, add lysozyme to 1 mg/ml and incubate on ice for 30 min.

4. Sonicate on ice (1 min bursts/1 min cooling/200-300 Watt) and monitor cell breakage by measuring the release of nucleic acids at A_{260} until it reaches a maximum.

5. If the lysate is very viscous, add RNase A (10 µg/ml) and DNase I (5µg/ml), and incubate on ice for 10 - 15 min. Alternatively, draw the lysate through a narrow gauge syringe needle several times.

6. Centrifuge at >10,000xg for 20 min, and collect the supernatant.

7. Add 8 ml of a 50% slurry of Ni-NTA resin, previously equilibrated in Sonication Buffer, and stir on ice for 60 min. Alternatively, load the lysate at 2 - 3 column volumes per hour onto a 4 ml Ni-NTA column, previously equilibrated with Sonication Buffer.

8. Load resin into a 1.6 cm diameter column, and wash with Sonication Buffer (flow rate 0.5 ml/min), until the A_{280} of the flow through is less than 0.01 (approx. 40 - 80 ml).

9. Wash the resin with Wash Buffer (50 mM Na-phosphate, 300 mM NaCl, 10% glycerol, pH 6.0), until the flow-through A_{280} is less than 0.01 (approx. 80 ml).

10. Elute the protein with a 30 ml gradient of 0 - 0.5 M imidazole in Wash Buffer. Collect 1 ml fractions, and analyze on SDS-PAGE. Do not boil samples which contain imidazole before SDS-PAGE, as it will partially hydrolyze acid labile bonds. Heat the sample for 10 min at 37°C immediately before loading the gel.

Alternate elution procedure
Follow standard protocol for steps 1 through 9.

10. Elute the protein with a 30 ml pH 6.0 to pH 4.0 gradient in Wash Buffer. Collect 1 ml fractions, and analyze on SDS-PAGE.

Note

a. If it is desirable to keep the pH above 7.0 at all times, the column can be washed with Sonication Buffer containing 0.8 - 40 mM imidazole, and eluted with a 50 ml gradient of 0 - 0.5 M imidazole in Sonication Buffer.

b. If preferred, elution can be performed using a step gradient. Most proteins will be efficiently eluted by Wash Buffer at pH 4.5, although many 6xHis proteins (particularly monomers) can be eluted at a higher pH. If elution at a higher pH is desired, most proteins can be efficiently removed from the resin with 250 mM imidazole in either Wash or Sonication Buffer.

c. The composition of the sonication, wash and elution buffers can be modified to suit the particular application e.g. by adding 0.5 - 1.0% Tween®-20, 5-10 mM ß-ME, 1 mM PMSF or increased NaCl or glycerol concentrations. (See Section VI.4.)

Protocol 6

Native Purification of Periplasmic Proteins

1. Grow and induce a 1 liter culture as described in Protocol 3.

2 Harvest the cells by centrifugation at 4000xg for 20 min. Resuspend the pellet in 30 mM Tris/HCl, 20% sucrose, pH 8.0, at 80 ml per gram wet weight of cells. Keep on ice and add EDTA dropwise to 1 mM. Incubate on ice for 5 - 10 min with gentle agitation.

3. Centrifuge at 8000xg for 20 min at 4°C, remove all the supernatant, and resuspend the pellet in the same volume of ice cold 5 mM $MgSO_4$. Shake or stir for 10 min in an ice bath.

4. Centrifuge at 8000xg for 20 min at 4°C. The supernatant is the cold osmotic shock fluid.

5. Dialyze extensively against Sonication Buffer before continuing with the purification to remove excess EDTA.

6. Process the cold osmotic shock fluid on Ni-NTA as described in Protocol 2, Step 7.

9. Purification of Proteins Under Denaturing Conditions

Most insoluble proteins can be solubilized in 6 M guanidine hydrochloride even if they have formed inclusion bodies. The protocol described here uses 6 M GuHCl as the solubilization agent for that reason. If a milder denaturant, such as 8 M urea is sufficient, that can be substituted in the procedure. Since proteases are also denatured, there is no need to perform denaturing purification in the cold. It should always be performed at room temperature, or there is risk that the denaturants will precipitate.

Protocol 7

Denaturing Purification of Insoluble Proteins

1. Grow and induce a 500 ml culture as described in Protocol 3.
2. Harvest the cells by centrifugation at 4000xg for 20 min. Store at -70°C if desired.
3. Thaw cells for 15 min and resuspend in Buffer A (6 M GuHCl, 0.1 M Na-phosphate, 0.01 M Tris/HCl, pH 8.0) at 5 ml per gram wet weight. Stir cells for 1 hr at room temperature.
4. Centrifuge lysate at 10,000xg for 15 min at 4°C. Collect supernatant.
5. Add 8 ml of a 50% slurry of Ni-NTA resin, previously equilibrated in Buffer A. Stir at room temperature for 45 min, then load resin carefully into a 1.6 cm diameter column. Alternatively load the lysate, at a flow rate of 10 - 15 ml/hr, onto a 4 ml Ni-NTA column pre-equilibrated in Buffer A.
6. Wash with 10 column volumes of Buffer A, and 5 column volumes of Buffer B (8 M urea, 0.1 M Na-phosphate, 0.01 M Tris/HCl, pH 8.0). If necessary, wash further until the A_{280} of the flow-through is < 0.01.
7. Wash with Buffer C (8 M urea, 0.1 M Na-phosphate, 0.01 M Tris/HCl, pH 6.3) until the A_{280} is < 0.01.
8. Elute the recombinant protein with 10 - 20 ml Buffer D (8 M urea, 0.1 M Na-phosphate, 0.01 M Tris, pH 5.9), followed by 10 - 20 ml Buffer E (8 M urea, 0.1 M Na-phosphate, 0.01 M Tris, pH 4.5). Collect 3 ml fractions from each elution, and analyze by SDS-PAGE.
9. Elute with 20 ml Buffer F (6 M GuHCl, 0.2 M acetic acid). Collect 3 ml fractions and analyze by SDS-PAGE.

Alternate elution procedure

Follow standard protocol for steps 1 through 7.

8. Elute the protein with 50 ml of Buffer C containing 250 mM imidazole. Collect 3 ml fractions and analyze by SDS-PAGE. Incubate at 37°C for 10 min instead of boiling to avoid imidazole mediated cleavage of labile peptide bonds.

Note

a. The purification is performed in 8 M urea because 6 M GuHCl precipitates in the presence of SDS, which makes SDS-PAGE analysis of samples difficult. There is no reason why either of these denaturants should not be used throughout the purification procedure.

b. Monomers usually elute in Buffer D, while multimers, aggregates and proteins with two 6xHis tags will generally elute in Buffer E.

c. Elution can also be carried out using a pH 6.5 - 4.0 gradient in 8 M urea, 0.1 M Na-phosphate, 0.01 M Tris/HCl.

d. Where possible, follow the chromatography by A_{280} and collect pools rather than fractions. Note that discolored or impure reagents may affect absorbance readings, and that low grade imidazole will also absorb light at 280 nm.

SDS-PAGE Analysis of Denatured Samples

On minigels it is usually sufficient to analyze 5 µl samples of each fraction in an equal volume of SDS-PAGE loading buffer, with or without 3% ß-ME. Since the fractions which contain GuHCl will precipitate with SDS, they must either be very dilute (1:6), dialyzed before analysis, or separated from the GuHCl by TCA precipitation. (Dilute samples to 100 µl, add equal volume of 10% TCA, leave on ice for 20 min. Spin 15 min in a microfuge, wash pellet with 100 µl of ice cold ethanol, dry and resuspend in sample buffer.) If there is any GuHCl present, samples must be loaded immediately after incubation for 7 min at 95°C.

APPENDIX A

Ni-NTA Resin

1. Specifications and Handling

Ni-NTA is supplied pre-swollen and charged with Ni^{2+} as an aqueous suspension (50%) containing 30% ethanol as a preservative. The fully charged resin exhibits a pale blue-green color due to the nickel ions. The Ni-NTA resin contains 8 - 12 µmol Ni^{2+}/ml gel and has a binding capacity of approximately 5 - 10 mg/ml resin for the protein DHFR-6xHis. The capacity of the resin may vary to some extent with the size and shape of the recombinant protein. For optimum results, we recommend flow rates not exceeding 3 - 4 column volumes per hour.

The resin is stable under a wide variety of conditions, and need not be stored cold, except in order to inhibit growth of microorganisms. For storage after use, NTA-resin should be washed with 0.2 M acetic acid, 30% glycerol and then rinsed in water. It should be stored in 30% ethanol to inhibit microbial growth. The resin can be temporarily stored for up to one week in any of the denaturing buffers.

Use only double-distilled water for buffers and solutions which will contact the Ni-NTA resin. Ionic impurities in the water may affect the Ni binding properties.

2. Preparation of Columns

The two sizes of columns supplied in the QIA*express* kit are appropriate for most lab-scale separations (column volume: 1 ml and 10 ml) and ensure consistently high quality results.

1. Mount the column and add 5 ml H_2O to check for leaks. Remove air from the bed support. Drain the column by removing the bottom cap, leaving about 1 cm H_2O.
2. Thoroughly resuspend the Ni-NTA resin, transfer the desired amount into a tube and allow it to settle. This can be achieved by centrifuging at low speed (500 - 600 rpm) for several minutes.
3. Mix the resin with 1 volume H_2O. (The slurry is diluted to prevent the retention of air bubbles.)
4. Carefully pour the slurry into the column.
5. Let the resin settle for 1 hr.
6. Remove excess H_2O, leaving about 1 cm H_2O above the resin.

7. Insert an upper frit into the column and push the frit into the H_2O in order to remove the air bubbles. Pack the resin by pushing the frit carefully onto the upper surface.

8. Open the bottom cap and drain the column. Columns containing the hydrophobic upper frit will not run dry. The flow will stop automatically as the buffer reaches the upper frit.

9. Wash the column with 5 volumes of H_2O.

10. Equilibrate with 10 volumes of the appropriate sample application buffer.

Note: If purification is to be carried out on a previously used column under denaturing conditions, a wash with Buffer F is recommended before equilibration.

3. Re-use of Ni-NTA Resin

The re-use of Ni-NTA resin depends on the nature of the sample and should only be performed with identical recombinant proteins. Based on the experience of Hoffmann-La Roche Ltd. (Basel, Switzerland), who have purified more than 150 different proteins on Ni-NTA resin, we recommend a maximum of 3 - 5 runs per column.

If the Ni-NTA-agarose changes from light blue-green to brownish-grey the following regeneration procedure is recommended.

Procedure:

1. Wash the column with 2 vol buffer F.

2. Wash the column with 2 vol H_2O.

3. Wash the column with 3 vol 2% SDS.

4. Wash the column with 1 vol 25% EtOH.

5. Wash the column with 1 vol 50% EtOH.

6. Wash the column with 1 vol 75% EtOH.

7. Wash the column with 5 vol 100% EtOH.

8. Wash the column with 1 vol 75% EtOH.

9. Wash the column with 1 vol 50% EtOH.
10. Wash the column with 1 vol 25% EtOH.
11. Wash the column with 1 vol H_2O.
12. Wash the column with 5 vol 100 mM EDTA, pH 8.0.
13. Wash the column with 2 vol H_2O.
14. Recharge the column with 2 vol 100 mM $NiSO_4$.
15. Wash the column with 2 vol H_2O.
16. Wash the column with 2 vol Buffer F.
17. Equilibrate with 2 vol Buffer A or Sonication Buffer.

Please note that the ethanol gradient (Steps 4 - 10) is designed to prevent precipitation of protein on the Ni-NTA column, and should not be omitted.

APPENDIX B

QIA*express* Trouble Shooting Guide

Proteins are extremely variable in their characteristics, and there are few guarantees about how they will behave in a given situation. Most proteins will be efficiently expressed in the QIA*express* system, and almost all proteins with a 6xHis affinity tag can be purified on Ni-NTA resin — optimal results, however, will require some input from the researcher.

This manual provides detailed information about how to optimize both the expression and purification parameters of the system for a particular protein. Please read it carefully. The following tips may also be of assistance. If you have any further questions about the QIA*express* system, please call our Technical Service Department.

1. Protein Expression

Slow growth of transformed cells

Expression is poorly repressed: Grow plasmids in a host strain containing pREP4. Try growing a different transformed colony, or reducing the growth temperature while growing the cells..

No or low expression

Conditions for expression are incorrect: Check expression of control plasmid.

Coding sequence is ligated into the incorrect reading frame: Sequence the ligated junctions.

Coding sequence is mutated (PCR cloning); contains internal start site; internal stop site; poorly used codons: Check sequence of insert fragment.
See Section III.4 - III.6 and Section IV.

Protein is insoluble: Check both cytoplasmic and insoluble fractions for protein (see Protocol 4). Try to solubilize protein with denaturants or detergents.

Protein is secreted: Remove all signal sequences from the coding region.

Protein is poorly expressed: See Section V of this manual for ways to improve expression levels.

Protein is being rapidly degraded: Make a time course to check the kinetics of growth and induction. If the protein is small (<10 kd), consider adding an N-terminal carrier protein such as DHFR (Section III.3). If degradation is occurring after cell lysis, consider adding protease inhibitors.

Formation of inclusion bodies

Expression level too high (protein can't fold correctly): Reduce expression levels by titrating the amount of IPTG added for induction (0.5 mM, 0.1 mM). Refer to Section V.6.

Protein is insoluble: See Section V for ways to enhance solubility of proteins by altering the growth and induction conditions.

Note: It may not be necessary to use denaturing conditions for purification if the protein of interest is insoluble or has formed inclusion bodies. Check the levels of soluble protein remaining in the cytoplasm. This may be purified with Ni-NTA resin under native conditions, and the insoluble portion purified under denaturing conditions.

Protein appears larger than expected on SDS gel

Some proteins with 6xHis tags attached run more slowly on SDS gels than equivalent untagged proteins, and may appear to be several kD larger than expected.

2. Protein Purification

Protein does not bind to the Ni-NTA resin

6xHis tag is not present: Sequence ligation junctions to ensure correct reading frame. Check for possible internal translation starts (N-terminal tag) or premature termination sites (C-terminal tag).

6xHis tag is 'hidden': Purify protein under denaturing conditions (low or high concentrations of urea as needed). Move tag to the other end of the protein.

6xHis tag has been degraded or removed: Check that the 6xHis tag is not attached to a portion of the protein that is processed after translation.

Binding conditions incorrect: Check pH and composition of all buffers and solutions. Ensure that there are no chelating or reducing agents present, and that the concentration of imidazole is not too high.

Protein precipitates during purification

Temperature is too low: Perform the purification at room temperature.

Protein is aggregating: Try adding solubilizing agents such as 0.1% Triton® X-100 or Tween®-20, 10 mM ß-ME, up to 1M NaCl, or stabilizing cofactors such as

Mg^{2+}. These may be required in all buffers in order to maintain solubility of the purified protein.

Protein elutes in the wash buffer

Wash stringency is too high: Lower the concentration of imidazole or increase the pH slightly.

6xHis tag is partially hidden: Reduce washing stringency. Purify under denaturing conditions.

Buffer conditions incorrect: Check pH and composition of wash buffer. Ensure that there are no chelating or reducing agents present, and that the concentration of imidazole is not too high.

Protein does not elute

Elution conditions too mild (protein may be in an aggregate or multimer form): Elute with a pH or imidazole gradient to determine the optimal elution conditions.

Protein has precipitated in the column: Elute under denaturing conditions. Try binding and eluting in a batch format to avoid high local concentrations of protein.

Protein elutes, with contaminating proteins.

Washing not stringent enough: Elute with a pH or imidazole gradient to determine the optimum washing conditions.

Column bed volume too large: Reduce the amount of Ni-NTA resin, to reduce the amount of non-specific binding.See Section VI.5.

Contaminants are linked to tagged protein: Add ß-ME to a maximum of 10 mM to reduce disulphide links.

Contaminants are associated with tagged protein: Increase salt and/or detergent levels, or add ethanol/glycerol to wash buffer (Section II.4) to disrupt non-specific interactions.

Contaminants are short forms of the tagged protein: Check for possible internal translation starts (N-terminal tag) or premature termination sites (C-terminal tag).

Contaminants have high affinity for the Ni^{2+} resin: If stringent washing conditions do not remove contaminants, a second chromatographic step on ion-exchange or gel filtration media may be useful.

Protein is difficult to refold

Drop in denaturant is too rapid: Try using smaller dilution steps during dialysis, or use FPLC® to create a linear gradient when renaturing on the column.

Protein is too concentrated: Increase protein dilution. Try refolding on the colomn.

Protein is poorly soluble: Try adding glycerol to 30%, up to 1 M NaCl, and low levels of non-ionic detergent to enhance protein solubility.

Appendix C

Media and solutions

Media:

TE	10 mM Tris/HCl, pH 8.0, 0.1 mM EDTA.
LB medium:	10 g bacto-tryptone, 5 g bacto-yeast extract and 5 g NaCl per liter.
2xYT medium:	16 g bacto-tryptone, 10 g bacto-yeast extract and 5 g NaCl per liter.
Super medium:	25 g bacto-tryptone, 15 g bacto-yeast extract and 5 g NaCl per liter.
Psi-broth:	LB medium, 4 mM $MgSO_4$, 10 mM KCl.

Solutions:

Note: Due to the dissociation of urea, the pH of Buffers B, C, D and E should be adjusted immediately prior to use. Do not autoclave.

Buffer A:	6 M guanidine hydrochloride, 0.1 M NaH_2PO_4, 0.01 M Tris, pH adjusted to 8.0 with NaOH.
Buffer B:	8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris, pH adjusted to 8.0 with NaOH.
Buffer C:	Same composition as buffer B, but pH adjusted to 6.3 with HCl.
Buffer D:	Same composition as buffer B, but pH adjusted to 5.9 with HCl.
Buffer E:	Same composition as buffer B, but pH adjusted to 4.5 with HCl.
Buffer F:	6 M guanidine hydrochloride, 0.2 M acetic acid.
Sonication buffer:	50 mM Na-phosphate pH 7.8, 300 mM NaCl (pH adjusted with NaOH).

0.1 M IPTG: 1.41 g IPTG (isopropyl-ß-D-thiogalactopyranoside); add H_2O to 50 ml, sterile filtered.

TFB1: 100 mM RbCl, 50 mM $MnCl_2$, 30 mM KAc, 10 mM $CaCl_2$, 15% glycerol, pH 5.8, sterile filtered.

TFB2: 10 mM MOPS, 10 mM RbCl, 75 mM $CaCl_2$, 15% glycerol, pH 8.0, autoclaved.

TNE: 100 mM NaCl, 50 mM Tris/HCl pH 7.5, 1 mM EDTA

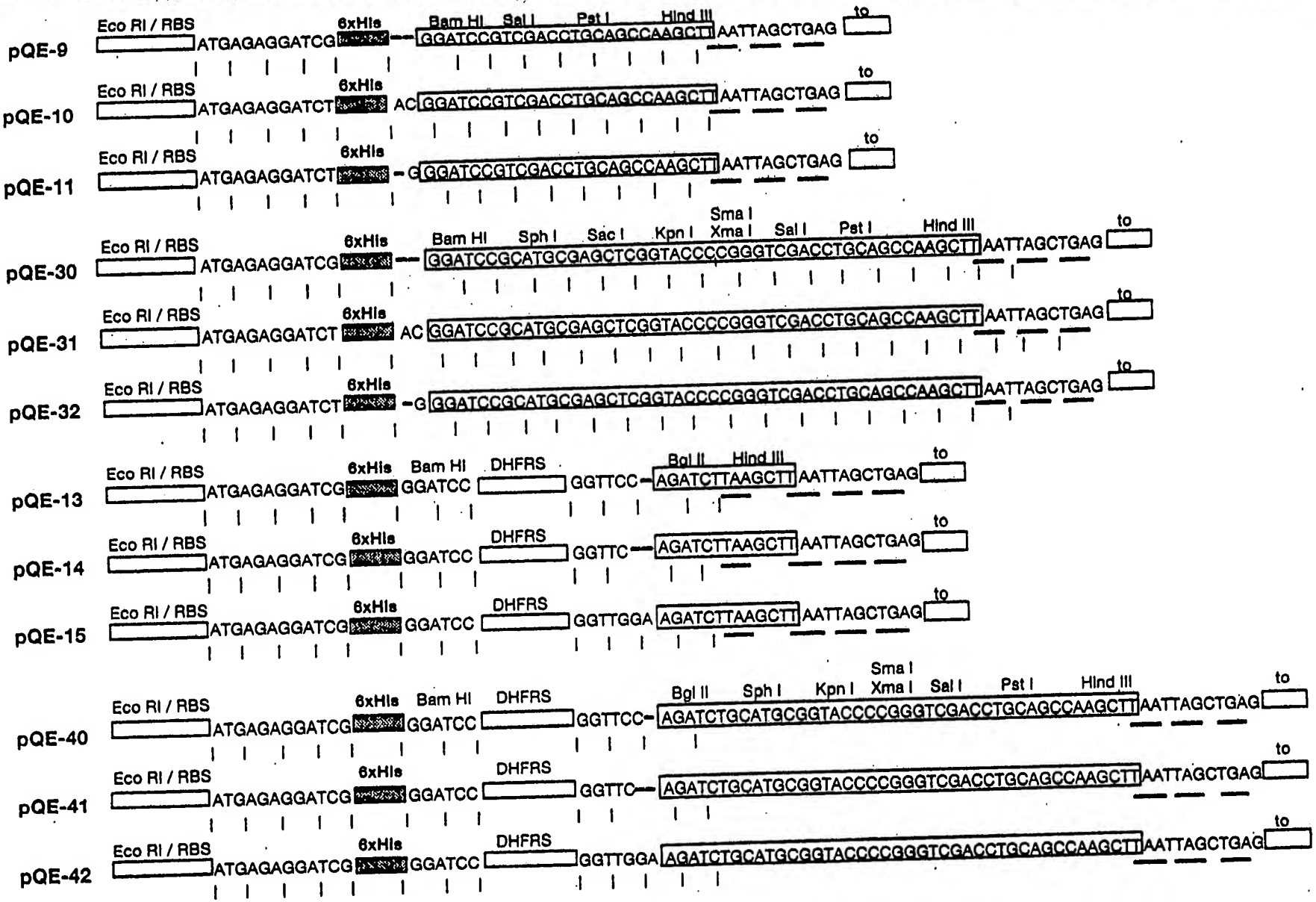
TE: 10 mM Tris/HCl pH 8.0, 0.1 mM EDTA.

5x SDS-PAGE Sample Buffer: 15% ß-ME, 15% SDS, 1.5% bromophenol blue, 50% glycerol.

APPENDIX D

Complete List of QIAexpress Vectors

The following list shows the variable region of all available pQE vectors. Those available as part of QIAexpress kits are shown in Section IV. The full sequence of all pQE vectors can be found on the accompanying disk. Restriction maps of prototype pQE vectors can be found in Appendix E.


pQE-9
Eco RI / RBS
ATGAGAGGATCG
6xHis
Bam HI Sal I Pst I Hind III
GGATCCGTCGACCTGCAGCCAAGCTT
AATTAGCTGAG
to
pQE-10
Eco RI / RBS
ATGAGAGGATCT
6xHis
AC
GGATCCGTCGACCTGCAGCCAAGCTT
AATTAGCTGAG
to
pQE-11
Eco RI / RBS
ATGAGAGGATCT
6xHis
G
GGATCCGTCGACCTGCAGCCAAGCTT
AATTAGCTGAG
to
pQE-30
Eco RI / RBS
ATGAGAGGATCG
6xHis
Bam HI Sph I Sac I Kpn I Sma I Xma I Sal I Pst I Hind III
GGATCCGCATGCGAGCTCGGTACCCCGGGTCGACCTGCAGCCAAGCTT
AATTAGCTGAG
to
pQE-31
Eco RI / RBS
ATGAGAGGATCT
6xHis
AC
GGATCCGCATGCGAGCTCGGTACCCCGGGTCGACCTGCAGCCAAGCTT
AATTAGCTGAG
to
pQE-32
Eco RI / RBS
ATGAGAGGATCT
6xHis
G
GGATCCGCATGCGAGCTCGGTACCCCGGGTCGACCTGCAGCCAAGCTT
AATTAGCTGAG
to
pQE-13
Eco RI / RBS
ATGAGAGGATCG
6xHis
Bam HI
GGATCC
DHFRS
GGTTCC
Bgl II Hind III
AGATCTTAAGCTT
AATTAGCTGAG
to
pQE-14
Eco RI / RBS
ATGAGAGGATCG
6xHis
GGATCC
DHFRS
GGTTC
AGATCTTAAGCTT
AATTAGCTGAG
to
pQE-15
Eco RI / RBS
ATGAGAGGATCG
6xHis
GGATCC
DHFRS
GGTTGGA
AGATCTTAAGCTT
AATTAGCTGAG
to
pQE-40
Eco RI / RBS
ATGAGAGGATCG
6xHis
Bam HI
GGATCC
DHFRS
GGTTCC
Bgl II Sph I Kpn I Sma I Xma I Sal I Pst I Hind III
AGATCTGCATGCGGTACCCCGGGTCGACCTGCAGCCAAGCTT
AATTAGCTGAG
to
pQE-41
Eco RI / RBS
ATGAGAGGATCG
6xHis
GGATCC
DHFRS
GGTTC
AGATCTGCATGCGGTACCCCGGGTCGACCTGCAGCCAAGCTT
AATTAGCTGAG
to
pQE-42
Eco RI / RBS
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GGATCC
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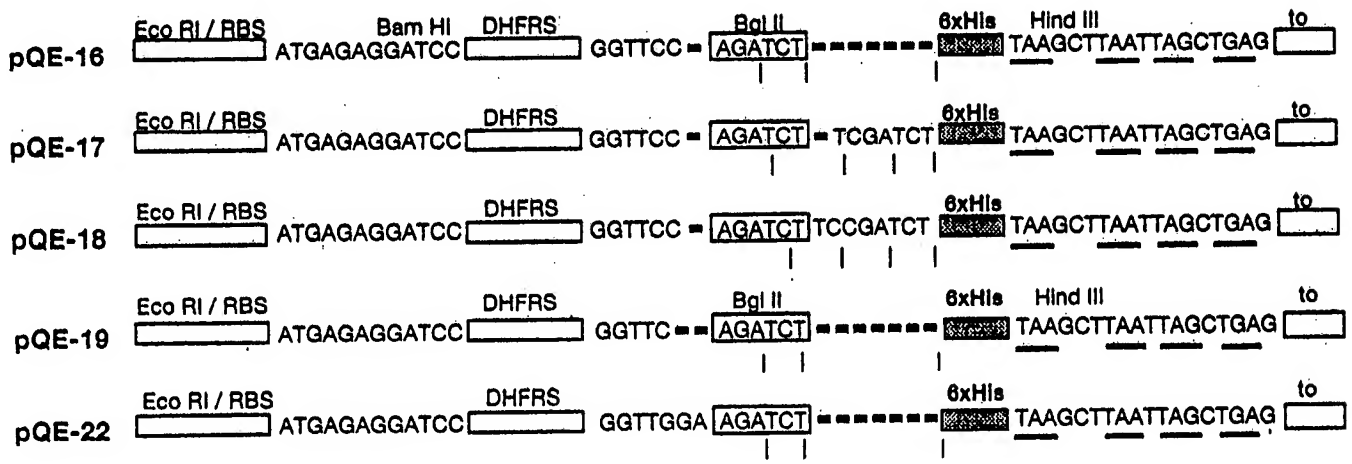
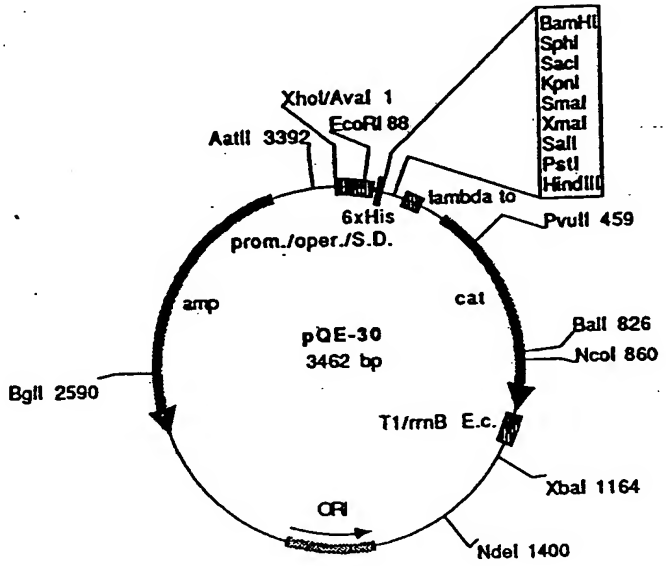
AATTAGCTGAG
to


pQE-16
Eco RI / RBS
ATGAGAGGATCC
Bam HI
DHFRS
GGTTCC
Bgl II
AGATCT
6xHis
Hind III
TAAGCTTAATTAGCTGAG
to
pQE-17
Eco RI / RBS
ATGAGAGGATCC
DHFRS
GGTTCC
AGATCT
TCGATCT
6xHis
TAAGCTTAATTAGCTGAG
to
pQE-18
Eco RI / RBS
ATGAGAGGATCC
DHFRS
GGTTCC
AGATCT
TCCGATCT
6xHis
TAAGCTTAATTAGCTGAG
to
pQE-19
Eco RI / RBS
ATGAGAGGATCC
DHFRS
GGTTC
Bgl II
AGATCT
6xHis
Hind III
TAAGCTTAATTAGCTGAG
to
pQE-22
Eco RI / RBS
ATGAGAGGATCC
DHFRS
GGTTGGA
AGATCT
6xHis
TAAGCTTAATTAGCTGAG
to

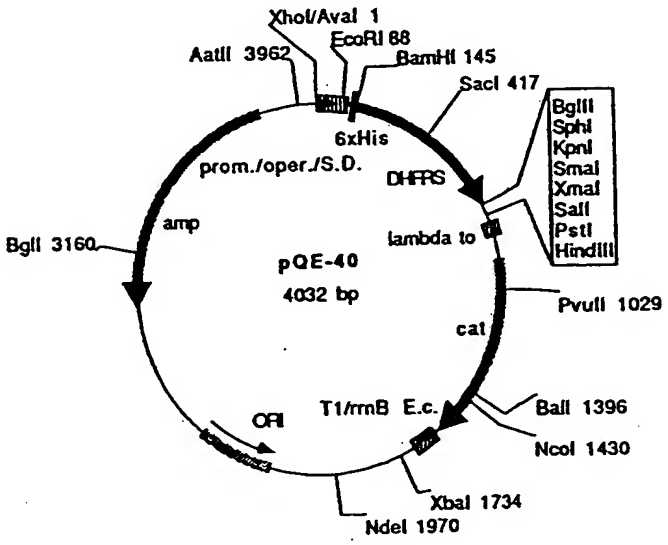
APPENDIX E

Restriction Maps of Prototype QIA*express* Vectors and pREP4

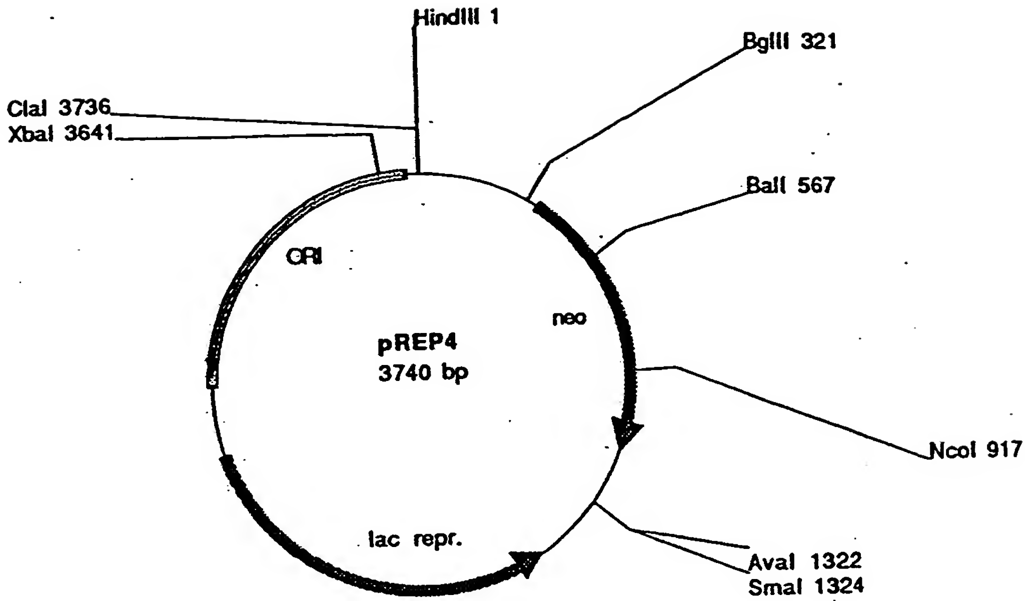
There are two types of pQE vectors — those that include the DHFR sequence (e.g. pQE-40) and those that do not (e.g. pQE-30). The restriction maps of the two prototype vectors are the same for all members of the same group, except for the variable cloning regions. The full sequences of all the pQE vectors can be found on the accompanying disk.



a) Restriction map of pQE-30.



b) Restriction map of pQE-40.



c) Restriction map of pREP-4.

```
            Xho I                                  operator I
  1   CTCGAGAAAT CATAAAAAAT TTATTTGCTT TGTGAGCGGA TAACAATTAT
                                                     TATA-Box

                              operator II             Eco RI
 51   AATAGATTCA ATTGTGAGCG GATAACAATT TCACACAGAA TTCATTAAAG
                 +1 start mRNA

                      ATG ->                6xHis
101   AGGAGAAATT AACTATGAGA GGATCGCATC ACCATCACCA TCACGGATCC
      RBS/SD                                          Bam HI

151   GCATGCGAGC TCGGTACCCC GGGTCGACCT GCAGCCAAGC TTAATTAGCT
        Sph I    Sac I    Kpn I   Sma I   Sal I   Pst I   Hind III
                                                      Stop 1 2 3

201   GAGCTTGGAC TCCTGTTGAT AGATCCAGTA ATGACCTCAG AACTCCATCT
```

d) Sequence of control region for pQE-30.

Restriction sites of pQE vectors

pQE vectors (- DHFRS)

Enzymes that do cut:

Aat II, Acc I, Afl III, Aha II, Alu I, Alw I, AlwN I, ApaL I, Asp700 I, Ava I, Ava II, Bal I, Bam HI, Ban I, Bbv I, Bbv II, Bgl I, Bsm I, Bsp1286, BspH I, BspM I, BspM II, Bsr I, BstN I, Cfr I, Cfr10 I, CviJ I, Dde I, Dpn I, Dra I, Dra II, Dsa I, Eco31 I, Eco57 I, Eco RI, Eco RII, Esp I, Fnu4H I, Fok I, Fsp I, Gdi II, Gsu I, Hae I, Hae II, Hae III, Hga I, HgiA I, HgiE II, Hha I, Hinc II, Hind III, Hinf I, Hin P1 I, Hpa II, Hph I, Ksp 632 I, Mae I, Mae II, Mae III, Mbo II, Mme I, Mnl I, Nci I, (Nco I), Nde I, Nhe I, Nla III, Nla IV, Nsp BII, Nsp HI, Pfl MI, Ple I, Pss I, (Pst I), Pvu I, Pvu, II, Rsa I, (Sal I), Sau 3a I, Sau96 I, Sca I, Sci I, SarF I, Sec I, SfaN I, Sso II, Ssp I, Sty I, Taq I, Taq II, Tha I, Tth111 I, Tth111 II,Xbal,Xca I, Xho I, Xho II.

Enzymes that do not cut:

Afl II, Aoc I, Apa I, Asp718 I, Asu II, Avr II, Ban II, Bbe I, Bcl I, (Bgl II), BssH II,BstE II, Bst X I, Cla I, Dra III, Eco47 III, Eco78 I, EcoN I, EcoR V, Fin I, Hpa I, (Kpn I), Mlu I, Nae I, Nar I, Not I, Nru I, Nsi I*, PmaC I, PpuM I, Rsr II, Sac I, Sac II, Sfi I, (Sma I), SnaB I, Spe I, (Sph I), Spl I, Stu I, (Xma I), Xma III.

*Nsi I cuts pQE-60 at the deleted Nco I site [829].

pQE vectors (+ DHFR)

Enzymes that do cut:

Aat II, Acc I, Afl II, Afl III, Aha II, Alu I, Alw I, AlwN I, ApaL I, Asp700 I, Ava I, Ava II, Bal I, Bam HI, Ban I, Ban II, Bbv I, Bbv II, Bgl I, Bgl II, Bsm I, Bsp1286, BspH I, BspM II, Bsr I, BstN I, BstX I, Cfr I, Cfr10 I, CviJ I, Dde I, Dpn I, Dra I, Dra II, Dsa I, Eco31 I, Eco57 I, EcoN I, Eco RI, Eco RII, Esp I, Fin I, Fnu4H I, Fok I, Fsp I, Gdi II, Gsul, Hae I, Hae II, Hae III, Hga I, HgiA I, HgiE II, Hha I, Hind III, Hinf I, Hin P1 I, Hpa II, Hph I, Ksp 632 I, Mae I, Mae II, Mae III, Mbo II, Mme I, Mni I, Nci I, Nco I, Nde I, Nhe I, Nla III, Nla IV, Nsp BII, Nsp HI, Pfl MI, Ple I, Pss I, Pvu I, Pvu, II, Rsa I, Sac I, Sau 3a I, Sau96 I, Sca I, Sci I, SarF I, Sec I, SfaN I, Sso II, Ssp I, Sty I, Taq I, Taq II, Tha I, Tth111 I, Tth111 II, Xba I, Xca I, Xho I, Xho II.

Enzymes that do not cut:

Aoc I, Apa I, Asp718 I, Asu II, Avr II, Bbe I, Bcl I, BspM I, BssH II, BstE II, Cla I, Dra III, Eco47 III, Eco78 I, EcoR V, Hinc II, Hpa I, (Kpn I), Mlu I, Nae I, Nar I, Not I, Nru I, Nsi I, PmaC I, PpuM I, (Pst I), Rsr II, Sac II, (Sal I), Sfi I, (Sma I), SnaB I, Spe I, (Sph I), Spl I, Stu I, (Xma I), Xma III.

p REP4

Enzymes that do cut:

Acc I, Afl III, Aha II, Alu I, Alw I, AlwN I, Aoc I, Apa I, ApaL I, Asp700 I, Asu II, Ava I, Ava II, Bal I, Ban I, Ban II, Bbe I, Bbv I, Bbv II, Bcl I, Bgl II, Bsp1286, BspM I, Bsr I, BssH II, BstE II, BstN I, Bst X I, Cfr I, Cfr10 I, Cla I, CviJ I, Dde I, Dpn I, Dsa I, Eco47 III, Eco57 I, Eco78 I, Eco RII, EcoR V, Fin I, Fnu4H I, Fok I, Fsp I, Gdi II, Gsu I, Hae I, Hae II, Hae III, Hga I, HgiA I, HgiE II, Hha I, Hinc II, Hind III, Hinf I, Hin P1 I, Hpa I, Hpa II, Hph I, Ksp 632 I, Mae I, Mae II, Mae III, Mbo II, Mlu I, Mme I, Mnl I, Nae I, Nar I, Nci I, Nco I, Nhe I, Nla III, Nla IV, Nru I, Nsi I, Nsp BII, Nsp HI, Pfl MI, Ple I, Pst I, Pvu II, Rsa I, Rsr II, Sac II, Sal I, Sau 3a I, Sau96 I, SarF I, Sec I, SfaN I, Sma I, Sph I, Sso II, Ssp I, Sty I, Taq I, Taq II, Tha I, Tth111 I, Tth111 II, Xba I, Xca I, Xho II, Xma I, Xma

Enzymes that do not cut:

Aat II, Afl II, Asp718 I, Avr II, Bam HI, Bgl I, Bsm I, BspM II, BspH I, Dra I, Dra II, Dra III, Eco31 I, EcoN I, Eco RI, Esp I, Kpn I, Nde I, Not I, PmaC I, PpuM I, Pss I, Pvu I, Sac I, Sca I, Sci I, Sfi I, SnaB I, Spe I, Spl I, Stu I, Xho I.

( ) check for presence/absence of the restriction site in the multi-cloning region of each pQE-vector.

Appendix F

QIA*express* Sequencing Primers.

5'-GGCGTATCACGAGGCCCTTTCG-3' Primer - Promotor region

5'-GAATTCATTAAAGAGGAGAAA-3' Primer - Type III / Type IV

5'-GTATAAGTTTGAAGTCTACG-3' Primer - Type II

5'-CATTACTGGATCTATCAACAGG-3' Primer - Reverse sequencing

Appendix G

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QIA*express* Expression and Purification System

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Appendix H

Complete List of QIA*express* Products and Catalog Numbers.

Product	Description	Catalog Number
QIA*express* Vector Kits		
Each Kit contains 5 µg each of the appropriate pQE vectors; 1µg of plasmid pREP4; 1 µg of control expression plasmid; host strains M15[pREP4] and SG13009[pREP4]; 10 ml of Ni-NTA resin; disposable plastic columns; buffers; pQE sequence information (Mac format disk) and a comprehensive manual, The QIA*expressionist*.		
Vector Kit Type II	pQE-40, pQE-41, pQE-42	32129
Vector Kit Type III	pQE-50, pQE-51, pQE-52, pQE-16, pQE-17, pQE-18	32139
Vector Kit Type IV	pQE-30, pQE-31, pQE-32	32149
Vector Kit Type SRF	pQE-8, pQE-12	32159
Vector Kit Type ATG	pQE-60, pQE-70	32169
QIA*express* Vector DNA		
pQE-3	25 µg (lyophilized)	33033
PQE-4	25 µg (lyophilized)	33043
pQE-5	25 µg (lyophilized)	33053
pQE-6	25 µg (lyophilized)	33063
pQE-6/pQE-12	25 µg (lyophilized)	33069
pQE-7	25 µg (lyophilized)	33073
pQE-7/pQE-12	25 µg (lyophilized)	33079
pQE-8	25 µg (lyophilized)	33083
pQE-9	25 µg (lyophilized)	33093
pQE-10	25 µg (lyophilized)	33103
pQE-11	25 µg (lyophilized)	33113
pQE-12	25 µg (lyophilized)	33123
pQE-13	25 µg (lyophilized)	33133
pQE-14	25 µg (lyophilized)	33143
pQE-15	25 µg (lyophilized)	33153
pQE-16	25 µg (lyophilized)	33163
pQE-17	25 µg (lyophilized)	33173
pQE-18	25 µg (lyophilized)	33183
pQE-19	25 µg (lyophilized)	33193
pQE-22	25 µg (lyophilized)	33223
pQE-30	25 µg (lyophilized)	33303
pQE-31	25 µg (lyophilized)	33313

pQE-32	25 µg (lyophilized)	33323
pQE-40	25 µg (lyophilized)	33403
pQE-41	25 µg (lyophilized)	33413
pQE-42	25 µg (lyophilized)	33423
pQE-50	25 µg (lyophilized)	33503
pQE-51	25 µg (lyophilized)	33513
pQE-52	25 µg (lyophilized)	33523
pQE-60	25 µg (lyophilized)	33603
pQE-70	25 µg (lyophilized)	33703

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Ready-to-use Ni-charged resin for batch or column chromatography.

Ni-NTA agarose	25 ml	30210
Ni-NTA agarose	100 ml	30230
Ni-NTA agarose	500 ml	30250

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Primer TypeII/IV	0.1 A260	34020
Primer Type III	0.1 A260	34030
Primer reverse sequencing	0.1 A260	34040

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M15[pREP4]*	stab culture	34211
SG13009[pREP4]*	stab culture	34964

QIA*express* Accessories

1 ml polypropylene disposable columns (50/pack)	34924
5 ml polypropylene disposable columns (50/pack)	34964

*Genotypes of the K12-strains M15[pREP4] and SG13009[pREP4]: Nal^S Str^S rif^S, lac^- ara gal mtl F^- $recA^+$ uvr^+.

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